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Experimental and Biochemical Basis for a Chemoprophylaxis and Chemotherapy of Virus Infections

By MATTHYS STAEHELIN

In spite of the extraordinary advances which have been made in the chemotherapy of bacterial diseases, chemotherapy of virus infections lags far behind, indeed, as far as clinical application is concerned, it has not even started Even the antibiotics with the widest therapeutic spectra have so far yielded only disappointing results in the treatment of virus diseases Exceptions are the larger viruses of the Psittacosis lymphogranuloma group, which are susceptible to many antibacterial drugs These organisms, however, are now classed separately as Chlamydozoaceae, together with the trachoma group and the coccoid agents responsible for conjunctivitis in cattle and poultry (WEISS, 1955) and are considered to be a special class between the Rickettsiae and the "True Viruses" The latter have so far been found to be extremely resistant to antibacterial chemotherapy

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* This review was written during tenure as a guest worker at the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

The main reason for this discrepancy seems to lie in the different natures of the infecting agent Pathogenic bacteria are independent organisms which can also grow in an appropriate cell free medium and which are only dependent on the host as far as the supply of nutrients is concerned Their metabolism also differs in many ways from that of the host Viruses on the other hand multiply only in living cells and are entirely dependent for their growth on the enzymatic activity of the host cell Furthermore, they differ only very little chemically from the normal tissue constituents, and particles of similar size and composition are also found in uninfected cells (CHAO ET AL, 1956)

A recent article entitled 'Antiviral Chemotherapy Current Status" begins as follows

"Since 1949 we have accumulated, without any special effort except observation more than 1100 references to virus chemotherapy None has been the crucial paper, that is, none has reported a really satisfactory agent " (CUTTING AND FURST, 1958)

Although it is true that no antivirus drug so far has found application beyond the laboratory level, it has to be appreciated that quite a number of substances have been found which not only inhibit the multiplication of virus in tissue culture but which also alter the course of an infection in animals and, in some cases, save the animals from certain death due to the infection with many times the lethal dose of a virus Enough data have accumulated to distinguish different mechanisms by which drugs can act and also to evaluate the criteria desirable in a successful drug

A survey of the more recent findings in virus chemotherapy will be given in a later part of this review Research so far has proceeded along two main lines (a) an empirical approach with systematic screening of as many compounds as possible and (b) a so-called "rational" approach For a closer understanding of the latter, it is perhaps pertinent to recall briefly our present knowledge on the biochemistry of viruses and of the processes leading to virus synthesis

Biochemistry of Viruses

All viruses contain protein and nucleic acid (KNIGHT, 1954) The smaller ones like poliomyelitis virus contain only these two components and are ribonucleoproteins The myxoviruses, e g influenza, mumps,

Newcastle Disease virus, etc, contain polysaccharides and lipids in addition The larger animal viruses like vaccinia are even more complex in their structure (SCHRAMM, 1958a)

The nucleic acid is of paramount importance since it has been found to carry the infectivity in a number of viruses even after its isolation from the intact virus This subject has been reviewed in the first volume of this series (COLTER, 1958), but several new reports on infectious nucleic acids from animal viruses have since appeared (ALEXANDER ET AL 1958, HUPERT ET AL, 1958, BROWN ET AL, 1958)

FRISCH NIGGEMEYER (1956) has made the extremely important observation that the ribonucleic acid content of a single virus is constant in a large number of viruses regardless of their size and weight The amount of nucleic acid in all viruses examined corresponded to a molecular weight of ca 2 000'000 In the case of tobacco mosaic virus, furthermore, there are indications that this material is present as one single molecule (GIERER, 1957, SCHUSTER ET AL, 1958) The genetic principle of virus infectivity would then be represented by one single molecule of 2 000'000 molecular weight Any process which prevents or alters only slightly the synthesis of this very large molecule might therefore exert a marked effect on virus reproduction This is indicated by the high activity which some purine and pyrimidine analogues exert on the multiplication of viruses Similarly, any chemical reaction with this large molecule might readily cause inactivation It has been found that the nucleic acid of tobacco mosaic virus is very susceptible to the action of formaldehyde and glyoxal derivatives, and 50% inactivation of the infectivity of the free nucleic acid was observed when only 12–15 molecules of formaldehyde or glyoxal derivatives had reacted with the amount of nucleic acid present in one virus particle (STAEHELIN, 1958, 1959)

Processes during Virus Synthesis

Very little is known about the processes which lead to the production of virus inside the cell For several viruses, it has been shown that first an adsorption of the virus to the cell has to take place which apparently is brought about by electrostatic forces and may be reversible under certain conditions (TOLMACH, 1957) In the case of tobacco mosaic virus, it is known that nucleic acid is set free on the surface or inside the cell and that the infection goes through a ribo-

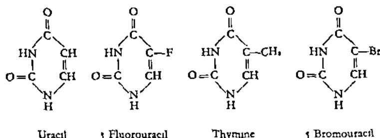
over the inhibition of protein synthesis The size of the nucleic acid molecule is considerably greater than that of the protein subunits, over 100 times in the case of tobacco mosaic virus (SCHRAMM, 1958) Only one molecule in each virus particle has therefore to be affected as against the much larger number of protein subunits (ca 2000 in tobacco mosaic virus, RAMACHANDRAN, 1958)

For the ribonucleic acid containing viruses it would seem important to inhibit specifically the synthesis of ribonucleic acid (RNA) without affecting the metabolism of deoxyribonucleic acid (DNA) RNA differs in several ways chemically from DNA, and the following characteristics can be used for the development of specific RNA inhibitors

(a) Ribose Many purine and pyrimidine analogues inhibit both RNA and DNA synthesis in general Substitution with either ribose or deoxyribose to the riboside or deoxyriboside can direct their activity preferentially towards the inhibition of one nucleic acid according to the closer resemblance with the respective precursors The significance of this structural specificity of the sugar component for the inhibition of virus synthesis can be seen for instance in the finding that the riboside of 5 Fluorouracil is a much more effective inhibitor of the growth of tobacco mosaic virus than its deoxyriboside (STAEHELIN AND GORDON, 1959) TAMM (1956) has also produced evidence that ribosides of halogenated benzimidazoles are more selective inhibitors of influenza virus multiplication than the free benzimidazoles or their deoxyribosides β D ribofuranosides were found to be active as inhibitors of virus growth at much lower concentrations than the free benzimidazoles and, in addition the inhibitory concentration of the ribosides proved to be a much smaller fraction of the toxic dose than was the case with free benzimidazoles or their deoxyribosides

(b) Uracil Another difference is the base composition since uracil

found that the size of the halogen substituent determines the charac of the compound The van der Waals radii of fluorine and bromine resembling closely those of hydrogen and the methyl group respectively, the size and shape of 5 Bromouracil is very similar to that of thymine, and that of 5 Fluorouracil similar to that of uracil



Uracil 5 Fluorouracil Thymine 5 Bromouracil

5-Bromouracil, especially in the form of its deoxyriboside, was found to be an inhibitor of the synthesis of bacteriophages which contain DNA (COHEN AND BARNER, 1956), but was without effect on the synthesis of tobacco mosaic virus (STAEHELIN AND GORDON, 1959) 5-Fluorouracil, on the other hand, inhibited the growth of this virus, and its action was reversed by uridine but not by thymidine

Similarly, thiouracil, an antagonist of uracil, is one of the most potent inhibitors of tobacco mosaic virus synthesis (COMMONER ET AL., 1952) Its effect is reversed by uracil Specific inhibition of RNA synthesis is strongly suggested since the inhibition of bacterial growth by thiouracil is reversed by uracil and not by thymine in contrast to some other uracil analogues (PULESTON ET AL., 1955)

(c) *Reactivity of the bases* The purine and pyrimidine bases of DNA are involved in the strong interstrand hydrogen bonds of the double stranded helix and are very unreactive In RNA, however, the amino groups of the bases are very reactive (STAEHELIN, 1958) and this, in accord with other observations (SCHRAMM, 1958b), is strong evidence that RNA is a free, single stranded molecule This difference in the reactivity of the bases has also some bearing on the effect of various antivirus substances Dicarbonyl compounds which inhibit the growth of influenza and other viruses in eggs, react specifically with the guanine groups of RNA (STAEHELIN, 1959), but not of DNA Only

whereas DNA does not (STAEHELIN, 1958) The chemical reaction of the virus nucleic acid which leads to its inactivation can also take place in the intact virus although somewhat stronger conditions are usually required But since the virus protein and the virus nucleic acid are

Although a chick embryo represents an intact organism, many *more substances have* been found active in eggs than in intact animals The following reasons have been suggested by HINUMA ET AL (1958a)

(a) The injection follows usually at the same place where the virus is given

(b) The substance is often given together with the virus

(c) An embryonated egg is a closed system with no excretory mechanism

For these reasons, drugs could have much easier access *to the virus* and the sites of virus synthesis and might also remain active for a much longer period of time than in the hatched animal But peculiarities in the metabolism and resistance of embryonic cells in general might also be of importance

4 *Animals* The crucial test for a compound is the proof of its therapeutic activity in an infected animal and here many compounds which show activity in tissue culture or eggs fail to show an effect Mice are usually used as test animals and some compounds have shown activity in monkeys Differences in the susceptibility of various animals have been described Thiosemicarbazones inhibit the growth of vaccinia in mice but not in rabbits (THOMPSON ET AL, 1953a) Mepacrine was found effective only in mice and young rats but not in any other animal tested (HURST, 1957) Netropsin protected mice inoculated intracerebrally with vaccinia virus but failed to prevent the development of lesions at the site of virus *inoculation in the skin* of rabbits (SCHABEL, 1953) The frequently observed higher susceptibility of mice might be due to the lower toxicity which some compounds exhibit towards mice so that they can be used at relatively higher concentrations than in other animals

Differences have sometimes also been noted according to the route by which the virus is given 2,6 Diaminopurine was found to be active against intraperitoneal and subcutaneous injection of Russian Spring and Summer Encephalitis but not against intracerebral injection (MOORE AND FRIEND, 1951) Xerosine was active against intracerebral but not against intravenous injection of influenza virus (GROUPE, 1954)

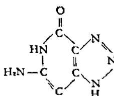
Compounds with Chemoprophylactic or Chemotherapeutic Activity

In view of the extremely large number of reports on virus chemotherapy, no attempt will be made toward completeness Several

excellent reviews have appeared on this subject during the past years (MATTHEWS AND SMITH, 1955, HURST AND HULL, 1956, HORSFALL AND TAMM, 1957, HURST, 1957). The compounds described hereafter were chosen as representatives of certain chemical groups, or of various modes of action, or because of the unique properties exhibited by some. Consideration has been given particularly to compounds which have shown activity in animals. The chemotherapy of bacteriophage infections although covering a quite extensive literature was omitted entirely because of the great dissimilarity in the chemistry, structure and sensitivity between animal and bacterial viruses.

Purines and Pyrimidines

Although purine and pyrimidine analogues have been proposed as antivirus drugs for some time, the theoretical basis for their use is now stronger than ever because of the reasons mentioned above. Analogues can act in two ways: (a) they can inhibit the enzymatic steps leading to the synthesis of the immediate precursors of nucleic acid, and (b) their incorporation into the newly formed virus can decrease the infectivity of this virus which then reduces the chances of further infection of new cells. The latter can best be seen in the spreading of a systemic infection in plants. At least three analogues—8 Azaguanine (MATTHEWS AND SMITH, 1955), 2 Thiouracil (JEENER ET AL. 1953), and 5 Fluorouracil (GORDON AND STAEHELIN, 1958)—were found to be incorporated into the nucleic acid of tobacco mosaic virus grown in their presence. In all cases the virus containing the analogue was found to have a decreased ability to initiate an infection as compared to a normal control.

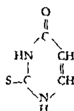


8 Azaguanine

8 Azaguanine (Guanozolo). MATTHEWS (1953) found this compound to be the most active inhibitor of tobacco mosaic virus among a series of purine and pyrimidine analogues tested by spraying on

infected leaves It was only active up to two days after infection and not after systemic infection had begun It was also active in turnip yellow mosaic virus in young plants although no activity was observed in older plants The activity was attributed to its being incorporated into the nucleic acid (MATTHEWS AND SMITH, 1955) since the only other active analogues, 8 Azaadenine and 4(5) Amino 1H 1,2,3 Triazolo 5(4) carboxamide were also incorporated Tobacco mosaic virus in which part of its guanine had been replaced by 8 Azaguanine was found to be only half as infective as normal TMV

8 Azaguanine also spared mice from otherwise fatal intracerebral infection with the virus of lymphocytic choriomeningitis (HAAS AND STEWART, 1956)



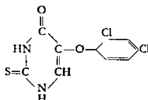
2 Thiouracil

Thiouracils COMMONER AND MERCER (1951) first described the inhibition of growth of tobacco mosaic virus by this compound The effect was reversed by uracil (COMMONER AND MERCER 1952) but only during the early stages of infection (JEENER, 1957) Similar effects were observed with 2 Thiocytosine and 2 Thiothymine (MERCER ET AL, 1953) 2 Thiouracil was incorporated to a considerable extent, 10–20% of uracil being replaced by the analogue The incorporated thiouracil was reported not to replace uracil randomly but to be preferentially located in terminal positions along the polynucleotide chain (MANDEL ET AL., 1957) These findings raise some doubts, since the number of endgroups in tobacco mosaic virus nucleic acid is extremely small and since erroneous results resembling endgroups have been described which were caused by impurities not related to the virus (MATTHEWS AND SMITH, 1957)

2 Thiouracil, as well as 2 Thio 4 Phenyl 6 oxy pyrimidine, 4 6 Diamino-5 nitro 2 thio pyrimidine and 5 Methyl 2 thiouracil caused complete suppression of the growth of poliomyelitis virus in monkey testicular cultures for a period up to 12 days (KNOX ET AL, 1957) In monkey kidney cultures, growth was delayed 24 hours and then

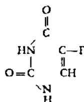
approached within 1-4 5 log units the virus titer obtained in untreated controls

An examination of the susceptibility of various types of polio myelitis virus led to the interesting result that higher concentrations of Thiouracil and 5 Methyl thiouracil were required to inhibit type 1 than type 2 or 3 virus In general, the effect could be overcome by the addition of natural pyrimidines



5 (2 4 Dichlorophenoxy) thiouracil

Dichlorophenoxythiouracil was found by THOMPSON ET AL (1951a) to be the most effective among a series of Phenoxythiouracils in protecting mice against intranasal or intracerebral infection with vaccinia Treated animals seemed to have a lower titer of virus in the brain than untreated controls The compound was effective when given intraperitoneally or when given with the diet and was less effective when administered 2 days after infection (MINTON ET AL, 1953)



5 Fluorouracil

5 Fluorouracil 5-Fluorouracil and especially its riboside, 5 Fluorouridine, inhibited the growth of tobacco mosaic virus in leaf discs (GORDON AND STAEHELIN 1959) The virus which grows in the presence of 5 Fluorouracil shows probably the utmost in incorporation of an analogue ever found in ribonucleic acid, about one third of its

uracil being replaced by 5 Fluorouracil Fig 2 shows the electrophoretic separation of an alkaline digest of the nucleic acid from tobacco mosaic virus grown in the presence of 5-Fluorouracil 5-Fluorouridylic acid can be seen moving ahead of the other nucleotides



Fig 2 Incorporation of 5 fluorouracil into the nucleic acid of tobacco mosaic virus (from GORDON AND STAEHELIN, 1959)

Electrophoresis of alkaline hydrolyzates in borate buffer pH 9 2

A Nucleic acid of virus grown in fluorouracil

B Nucleic acid of control TMV

The virus containing the analogue was found to have peculiar characteristics in regard to its infectivity Applied to *Nicotiana xanthia*, a local lesion host, it was found to cause exactly the same number of local lesions as did a normal virus In *Nicotiana tabacum*, however, in which the virus causes a systemic infection, much less

virus is produced at a given time as compared with the yield from normal virus (Fig 3) From these experiments, it was concluded that while containing an equal number of infecting units, the ability of the virus to initiate an infection was impaired (STAEHELIN AND GORDON, 1958) A similar phenomenon has been reported for a virus containing thiouracil (JEENER, 1957) The action of 5 Fluorouracil was not specific for the virus since it also affected the RNA metabolism of non infected plants (STAEHELIN AND GORDON, 1959)

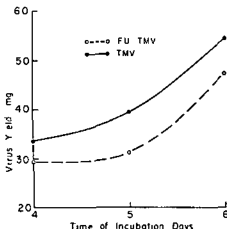
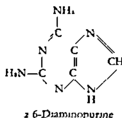


Fig 3 Yield of virus after infection with tobacco mosaic virus containing 5 fluorouracil (from GORDON AND STAEHELIN 1959)

5 Fluorouracil also prolonged the life of mice infected intra cerebrally with the virus of lymphocytic choriomeningitis (LEVY AND HAAS, 1958)

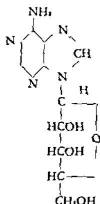


2 6-Diaminopurine

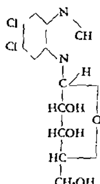
2,6 Diaminopurine This was the most inhibitory among a number of compounds tested by THOMPSON ET AL (1950) on the growth of vaccinia virus in minced embryonic tissue It also inhibited the MEF₁

(Lansing) strain of poliomyelitis in tissue culture (BROWN, 1952) and Russian Summer and Spring encephalitis in minced embryonic chicken or mouse tissue culture (FRIEND, 1951) With the latter virus, it was also effective in mice intraperitoneally *and increased about* threefold the percentage of mice surviving after intraperitoneal or subcutaneous infection, although no effect was observed on intra cerebral infection (MOORE AND FRIEND, 1951)

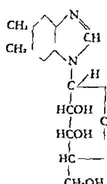
Benzimidazole Derivatives



Adenosine



5 6 Dichloro-1 β D ribo furanosyl benzimidazole (DRB)



5 6 Dimethylbenzimidazole riboside (in Vit B_{12})

The studies of TAMM and his associates on benzimidazole derivatives represent an extremely interesting example of the development of derivatives with more desirable properties from a basic component (TAMM, 1956a) Halogenated and alkylated benzimidazoles, originally considered as antagonists of 5,6 Dimethyl benzimidazole, a component of Vit B_{12}, were found to be more active inhibitors of the growth of influenza virus than benzimidazole, and their ribosides proved to be even more powerful TAMM (1956b) also compared the concentrations which caused macroscopic damage and reduction in oxygen uptake to the chorioallantoic membrane of embryonated eggs *in vitro* with the concentrations necessary to inhibit multiplication of influenza B virus (Fig 4) The toxic concentration of benzimidazole was found to be three times higher than the inhibitory concentration β D Ribofuranosyl derivatives were not only active at much lower

concentrations but also more "selective", i e the ratio between the toxic and inhibitory concentrations was twice as high with 5,6-Dichloro β D ribofuranosyl benzimidazole (DRB) and six times as high with 4 5,6-Trichloro β D ribofuranosyl benzimidazole (TRB) as with free benzimidazole This "selective" action was specific for the β ribofuranoside, neither the β-D ribopyranoside nor the arabinoside showed any higher selectivity than the free base The β D ribofuranosyl form being the natural form in which nucleotides are present in ribonucleic acid (RNA), this might indicate that the "selective" action is due to a specific inhibition of RNA synthesis with less inhibitory effects on other functions of purine bases, e g deoxyribonucleic acid synthesis The inhibition of the incorporation of orotic acid into ribonucleic acid by DRB (ALLFREY, 1957) as well as the finding that adenosine blocks the action of DRB on influenza virus (TAMM AND OVERMAN, 1957) corroborate this assumption Also, with vaccinia virus which contains DNA rather than RNA, the ribosides were found to be no more selective than the free bases (TAMM AND OVERMAN, 1957) when the virus was grown in chorio allantoic membranes

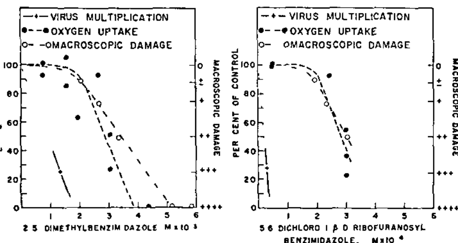


Fig 4 Virus inhibition and toxicity on chorioallantoic membranes (from TAMM, 1956)

Surprisingly, however, TAMM AND NEMES (1957) found no selective effect of DRB on the growth of poliomyelitis virus although

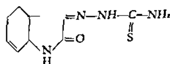
the latter contains only ribonucleic acid With this virus, the only compound which showed a more "selective" action than benzimidazole was 5,6 Dichloro D arabinosyl benzimidazole No clear relation ship, therefore, seems to exist between the type of nucleic acid of the virus and the selective action of β-D ribofuranosides Monkey cells being used in these experiments as compared to chorioallantoic membranes in the experiments with the influenza virus, the question arises whether the selectivity might be dependent on the host rather than on the virus

But the nature of the virus also seems to be of importance since with another benzimidazole derivative, 5 Methyl 2-D ribobenzimidazole, TAMM (1956c) observed an enhancement of the growth of influenza virus and an inhibition of the growth of vaccinia in the same host (chorioallantoic membrane)

Benzimidazole derivatives showed also some activity in animals DRB prolonged the survival time of mice infected with human influenza virus without protecting them from eventual death (TAMM, 1956) Apparently, the compounds could not be used in sufficient amounts to cause marked virus inhibition for a period of one week or longer without damage to the host

KISSMAN ET AL (1955) synthetized 5,6-Dichloro-β-D ribofuranosyl benzimidazole (DRB) and found it not to inhibit the multiplication of poliomyelitis virus in HeLa cells in up to toxic concentrations DRB in their hands also changed neither the mortality nor the mean survival time of mice infected with the PR8 strain of influenza A virus In view of this discrepancy regarding the activity of DRB, it is hoped that experiments on the inhibitory activity of this interesting compound will be repeated in other laboratories

Thiosemicarbazones



Isatin thiosemicarbazone

The chemotherapeutic activity of thiosemicarbazones against vaccinia was first found by HAMRE ET AL (1951) p Aminobenzal

dehyde 3 thiosemicarbazone inoculated into the yolk sack prolonged the survival time of infected chicken embryos Oral or subcutaneous administration had also some protecting effect in mice infected with vaccinia intranasally Benzaldehyde thiosemicarbazone protected a large number of mice when fed with the diet and was also active when the virus was given by the cerebral route (THOMPSON ET AL, 1951b) Several thiosemicarbazones of heterocyclic compounds were also found to be active by MINTON ET AL (1953) THOMPSON ET AL (1953) concluded that two properties are necessary to obtain a high degree of antiviral activity (a) the =N—NH—CS NH_2 group as such and (b) the presence of a cyclic component Isatin and 5 nitro 2 thenaldehyde were also effective when administered two days after infection (MINTON ET AL., 1953) The activity seemed to be specific against vaccinia and specific for mice No activity was found against St Louis encephalitis, Herpes, or Semliki Forest virus, nor was isatin thiosemicarbazone active against vaccinia in rabbits (THOMPSON ET AL., 1953a) EATON ET AL. (1951), however, reported that p nitro and p acetamid benzaldehyde thiosemicarbazones suppressed the production of pulmonary lesions in cotton rats inoculated intranasally with the virus of primary atypical pneumonia.

BAUER (1955) reexamined the activity of isatin thiosemicarbazone against the neurotropic HID strain of vaccinia A single dose of the insoluble compound suspended with the aid of gum arabic given subcutaneously 18 hours after infection was found to be superior to a single dose 3 hours after infection and gave complete protection against 100–1000 LD_{50} of the virus In addition, there was a synergic effect of isatin thiosemicarbazone and certain phenoxy thiouracils, notably 5 (2',4 Dichlorophenoxy thiouracil, since the combined effect of small doses of each was much greater than the individual effects of either compound given separately The virus titer in the brains of treated mice was usually one log unit below that in untreated mice, but apparently high enough to cause the formation of antibodies since mice surviving vaccine infection as a result of treatment with isatin thiosemicarbazone were immune to reinfection Apart from a slight effect against Rift Valley fever, BAUER also found no effect against other viruses His results were confirmed by BOCK (1956) who reported subcutaneous injection to be a superior mode of application compared with intraperitoneal injection The effect was also found to be restricted to mice, no effect being seen in rabbits

LUM ET AL (1957) found some thiosemicarbazones active against

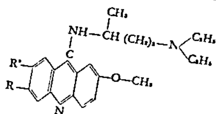
the PR 8 strain of influenza A virus in tissue cultures Interestingly, the activity was reversed by the addition of small amounts of Coenzyme A or pantothenic acid

The inhibition was believed to be competitive since the concentration of pantothenic acid was dependent on the concentration of thiosemicarbazone These experiments might help to elucidate the mode of action of thiosemicarbazones

Acridines

The studies of HURST and his associates (HURST, 1957, GREENHALGH ET AL, 1956) throw some light on interesting phenomena of virus and host specificity Among several acridines examined, the antimalarial drug mepacrine was found to possess very marked protective activities against a few viruses, especially against Equine encephalitis, Rift valley fever, and Louping ill (HURST ET AL, 1952) It was not active against a great number of other viruses A single oral dose of mepacrine given before or soon after virus infection protected a large number of mice even when high infecting doses of virus were used Growth of virus was wholly or partly suppressed according to the dose administered Deposits of a substance with the general properties of an acridine and presumably a metabolite of mepacrine appeared in the cells mainly of the reticuloendothelial system The action of mepacrine thus seemed to be somehow connected with these cells

The drug was active only in mice and adult rats, inactive in guinea pigs, rabbits, chickens, or monkeys HURST concluded that the active compound was presumably a metabolite The search for the latter led to most interesting results with various derivatives of mepacrine

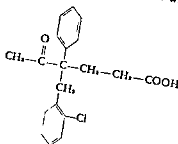


Mepacrine R = Cl R' = H
(Quinacrine, Atebrin)

Replacement of Chlorine by a nitro group ($R = NO_2$, $R' = H$) removed all activity for equine encephalitis, but this compound was active against psittacosis and lymphogranuloma. When the nitro group was shifted from the 6 to the 7 position in the acridine ring ($R = H$, $R' = NO_2$) activity was regained against equine encephalitis and lost against psittacosis and lymphogranuloma. None of three compounds had any activity against the virus *in vitro*. When the nitro group at position 7 was replaced by an amino group ($R = H$, $R' = NH_2$), all therapeutic activity against both large and small viruses was lost. The new compound, however, was remarkably active against all viruses *in vitro* and inactivated them in a very short time.

Caprochlorone

The effect of (levo γ-orthochlorobenzyl)-δ-oxo γ phenylcaproic acid (Caprochlorone) on the growth of PR 8 influenza virus was studied by Liu et al. (1957). It reduced the infectivity titer and the haemagglutinin titer in deembryonated eggs. Synthesis of the S antigen was less affected. This phenomenon is probably not a unique property of the drug since it was also found with DRB (Tamm, 1954). The compound was effective even when treatment was started as late as 20 hours after infection but the effect was most marked with earlier treatment.



Caprochlorone

For studies in mice, the compound was given in the form of the free acid suspended in 0.5% aqueous solution of carboxymethyl cellulose. Three to four mg were thus applied by gavage three times daily. When mice were infected with 6 MLD_{50} of PR 8 influenza

virus, treatment with caprochlorone resulted in survival of 19 out of 20 mice, whereas, 19 out of 27 control animals died The compound was less effective after infection with higher virus concentrations

After intranasal infection with 300 and 3000 MLD_{50} of virus, all animals survived upon combined treatment with caprochlorone and antiserum Either treatment alone protected less than half the animals at the higher infectious dose The combined treatment was also found to have an effect on the time of the onset of therapy. The combined treatment still protected 80% of the mice when applied only three days after infection, at which time either treatment alone had very little effect

The virus titer in lung suspensions was reduced by about one log unit upon treatment with Caprochlorone, the differences between treated and untreated mice being most marked two and three days after infection It was concluded that due to the treatment, a lethal concentration of virus was never reached but that sufficient amounts of virus were produced early to induce antibody formation in time to intercept virus formed later in the infection

Bacterial Polysaccharides

One of the smaller viruses, the pneumonia virus of mice, is susceptible to the action of a capsular polysaccharide from Bacillus Friedlander Other bacterial polysaccharides as well as blood group specific substances and ordinary agar agar also showed some effect (HORSFALL AND MCCARTHY, 1947) Intranasal instillation of 0 02 mg of polysaccharide from B Friedlander inhibited the multiplication of the virus in the lung and thus spared infected mice from otherwise certain death (HORSFALL, 1955) The bacterial polysaccharide still intercepted further virus multiplication when given up to four days after infection, at a time when almost maximal titer is reached GINSBERG AND HORSFALL (1951) have shown that this compound acts during the first half of the latent period of a multiplication cycle It therefore was most active during the first ten hours after infection, i e in the latent period of the first multiplication cycle Subsequent stages, however, were still affected by later application The polysaccharide was active against mumps but not against influenza A or B in mice or chicken Interestingly, the compound itself in amounts of 0 1 mg caused a temporary consolidation of the lung This effect did

not seem to be related to the virus inhibitory action. Oxydation with periodic acid greatly reduced its harmful action on the lungs without impairing its ability to inhibit multiplication of pneumonia virus of mice.

A different kind of bacterial polysaccharide was studied by MEIER ET AL. (1956). Intravenous injection of polysaccharides from gram negative bacteria decreased the death rate of mice infected with Columbia SK encephalomyelitis virus. The optimal time for injection was dependent on the amounts of polysaccharide which were injected and showed, at lower concentrations, a rather definite peak at 24 hours before infection (KRADOLFER ET AL., 1957). These polysaccharides did not inhibit the haemagglutination of influenza virus.

MACPHERSON ET AL. (1953), have found that NaOH treated polysaccharides from *Klebsiella aerogenes* and *Klebsiella cloacae* inhibited the haemagglutination of the influenza mumps Newcastle disease group of viruses by firm combination with red cells. No *in vivo* activity, however, has been found with the *Klebsiella* polysaccharides.

Antibiotics

The tetracyclines seem to be the therapy of choice against the psittacosis lymphogranuloma group of viruses (GREEN, 1950, FITZ ET AL., 1955, KATZ, 1956) and seem to be extremely effective in the therapy of psittacosis ornithosis in fowl (MEYER ET AL., 1955, COVER, 1957). This group of large viruses has distinctly different characteristics from those of the smaller "true viruses" and is now considered as a special class, Chlamydozoaceae, between viruses and rickettsiae. The chemotherapeutic susceptibility to sulfonamides and antibacterial antibiotics also puts these organisms in a class much closer to bacteria than to the smaller viruses. A number of different antibiotics, however, have been found which inhibit the growth of "true" viruses and also show protective effects in animals.

Netropsin. This antibiotic from *Streptomyces netropsis* given intraperitoneally the same day as intracerebral infection with vaccinia increased the survival rate in mice and led to immunity in the surviving animals (SCHABEL, 1953). The administration of Netropsin, however, resulted in marked loss of weight. It was inactive against any other virus tested.

Helenine. From a mold which grew on a photograph of his wife,

Helen, and which was classified as *Penicillium funiculosum*, SHOPE (1953a) obtained an antibiotic, appropriately named Helenine, which when given intraperitoneally 3 and 24 hours after infection increased the survival index in mice infected with Columbia SK virus The treatment with the antibiotic also delayed the entrance of virus into the brain It showed even greater therapeutic effect against Semliki forest virus (SHOPE, 1953 b) It was also found to reduce the morbidity in monkeys infected with poliomyelitis virus (COCHRAN ET AL, 1956) Treated animals which developed paralysis also had a three times longer incubation period Activity, however, was restricted to treatment during the time of incubation Attempts to demonstrate chemotherapeutic activity failed once the animals had a fever or showed signs of paralysis

M5-8450 Similar results were found with an antibiotic from *Penicillium stoloniferum* (COCHRAN ET AL, 1956) This antibiotic, M5 8450, has been shown by POWELL ET AL (1952) to have chemoprophylactic activity in mice against Semliki forest and MM viruses One single intraperitoneal dose given one day before infection gave full chemoprophylactic protection against 100 LD_{50} of MM virus injected subcutaneously but did not render the mice refractory to later infection (POWELL ET AL, 1952) It also afforded protection against the MEF, strain of poliomyelitis when antibiotic and virus were given intraperitoneally (POWELL ET AL, 1953)

Xerosine This antibiotic from Achromobacter shows some rather striking peculiarities Given subcutaneously, it was effective against intranasal infection with 10,000 LD_{50} of influenza A virus (GROUPE, 1952) and had still some effect in one single dose given 48 hours after infection Intraperitoneally, it was toxic, however, and orally without effect Using 10,000 LD_{50}, it delayed the development of pulmonary lesions in mice but did not prevent final death It also failed to affect the reproduction of virus in the lung or allantoic fluid

Development of pneumonia in mice following infection with mouse pneumonitis virus and influenza B virus was suppressed by daily parenteral injections of Xerosine Mortality following intracerebral but not intravenous inoculation of toxic doses of influenza A virus was reduced although Xerosine failed to affect viral synthesis *in vivo* and did not possess antiviral properties *in vitro*

Interestingly, Xerosine suppressed the development of non transmissable pneumonia due to Newcastle Disease virus in which apparently no virus multiplication occurs (GROUPE, 1953) indicat-

Table I Summary of the Mode of Action of Representative Antibiotics against Growth of PR 8 Virus

Group	Antibiotic	Direct inactivation	Prevention of adsorption	Effect of pretreatment of the membrane	TTC reaction	Possibility as inhibitor of intracellular virus growth
I	Cyclohexidine	++	—	—	—	—
	Thiolutin	+—	—	—	—	—
	Clavacin	++	—	++	+	—
	Bacillomycin	++	—	++	+	—
	Toyamycin	++	—	+	—	—
II	E 416 Substance	++	—	+—	—	—
	F 43 Substance	—	—	+—	—	—
	E 300 Substance	—	—	++	—	—
	Actinomycin	—	—	—	—	++
III	Myxoviromycin	—	—	—	—	++
	Streptothricin	—	—	—	—	++
	E 150 Substance	—	—	++	+	—
	Colistin	+—	—	+	—	—
IV	G 72 Substance	—	—	—	—	++
	Erythromycin	—	—	—	—	++
	Netropsin	—	—	—	—	++
	Chloramphenicol	—	—	—	—	++

(from MIYAKAWA ET AL., 1958)

that its activity is related to the reaction of the tissue rather than to virus multiplication

Myxoviromycin MIYAKAWA ET AL (1958) in an attempt to evaluate critically a large number of antibiotics have tested the effect of various antibiotics on the growth of the PR 8 strain of influenza A virus in tissue cultures of chorioallantoic membranes of chicken eggs Active compounds were further checked as to their direct inactivating action on free virus, prevention of adsorption of virus, and as to their toxicity on chorioallantoic membranes Glucose dehydrogenase activity as measured by macroscopic examination after incubation with Tri-

phenyltetrazolium chloride and the effect of pretreatment of the membranes with the antibiotic prior to infection were used as criteria of toxicity. As seen in Table I, some antifungal agents (group I) were found to be effective inhibitors of virus growth. Most of them showed direct virucidal activity. Active substances were also found among some antibiotics which were soluble in organic solvents with a high activity against gram positive bacteria (group II). Most of these, however, were toxic to animals. More promising agents were found among some basic water soluble antibiotics like streptomycin (group III) and some miscellaneous compounds (group IV).

Of thirteen antibiotics which were active in tissue culture, only seven reduced the haemagglutinin titer in embryonated eggs and only Myxoviromycin, a substance from Streptomyces sp. E 212, inhibited virus multiplication in the lungs of mice in non toxic concentrations (HINUMA ET AL., 1958a). The therapeutic effect of myxoviromycin was further studied in regards to survival rate of mice receiving 30–300 LD_{50} of influenza A virus (HINUMA ET AL., 1958b). The effect could greatly be enhanced by the additional injection of antiserum (Table II). Myxoviromycin alone was able to spare 50% of the animals receiving 30 LD_{50} of the virus. Upon combined treatment, all mice receiving 300 LD_{50} survived although neither treatment alone was very effective against this high dose of virus. It may be mentioned that similar synergic effects with antiserum have been reported by SHOPE against Columbia SK encephalitis with Helenine (SHOPE, 1953a).

Antibiotics from Actinomyces. Antivirus activity of several antibiotics from Actinomyces has recently been reported in the Russian literature*.

Violarine, an antibiotic from Actinomyces violaceus, caused a suppression of virus in mice infected with the virus of endemic encephalitis. It also suppressed the growth of influenza and vaccinia viruses in chick embryos (KRASILNIKOV ET AL., 1958).

Heliomycin, a crystalline product from Actinomyces flavochromogenes, var. heliomycini, saved to some extent mice which had been infected intranasally with influenza virus. The effect was only present when the antibiotic was given before or within three hours after infection, and only after infection with a minimal dose of virus (BRAZHNIKOVA ET AL., 1958).

* These reports were made available to the author through the courtesy of the Translating Unit of the National Institutes of Health, Bethesda, Maryland.

Table II Comparison of Effect of Myxoviromycin or Immune Serum alone with that of Combination of both Agents in Influenza Virus Infection in Mice

Group	Treatment Dose of therapeutic agent (per mouse)	Administration		Mortality of mice (%) Infecting dose per mouse of PR 8 virus			
		Route	Time	3 LD 50	30 LD 50	300 LD 50	3000 LD 50
1	1 mg of Myxoviromycin	s c	6 hours after infection	30	50	100	100
2	0 5 cc of immune serum	i p	immediately after infection	30	50	70	100
3	0 5 cc of immune serum and 1 mg of Myxoviromycin	i p s c	immediately after infection 6 hours after infection	0	0	0	40
4	none	—	—	70	100	100	100

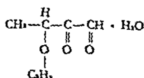
(from HINUMA ET AL 1958b)

Virusin 1609, an antibiotic from Actinomyces 1609, was active against vaccinia in chick embryos (GERMANOVA ET AL, 1957) 0 5 mg of the compound suppressed the infection completely It was inactive against Theiler's mouse encephalitis virus No virucidal effect was observed with either Heliomycin or Virusin 1609

Substances interacting with the Virus itself (Virucidal Agents)

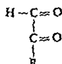
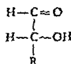
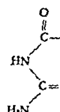
Dicarbonyl compounds Various glyoxal derivatives were found to inhibit the growth of influenza and other viruses in eggs (DE BOCK

ET AL., 1957, MCLIMANS ET AL., 1957) One of the most active compounds was Kethoxal (β-ethoxy α keto butyraldehyde) Injection of



Kethoxal

2 5 mg of the compound into the allantoic sack fifteen minutes before or a few hours after infection spared chick embryos from death due to 50 LD_{50} of Newcastle Disease virus and reduced the haemagglutinin titer after infection with influenza and mumps viruses In addition, the substances were also found to be virucidal Their activity was therefore attributed to their direct action on the virus MCLIMANS ET AL. (1957) made a study to determine the chemical configuration *responsible for this virucidal action It was found that all substances* having an aldehyde group adjacent to a carbonyl (I) or a secondary hydroxyl group (II) exerted a virucidal effect

I II III

It has been found that the same substances undergo a specific reaction with the guanine *groups of ribonucleic acid* (STAEHELIN, *1959*) This specificity was attributed to the diamine structure (III) which is present only in guanine among the bases of RNA This group offers the possibility *to form a stable ring with the two reactive groups* of the glyoxal derivative In a series of active and inactive glyoxal derivatives excellent agreement was found between the reaction with guanylic acid and the antiviral activity Only compounds which reacted with guanylic acid showed antivirus activity The compounds were found

to inactivate the free nucleic acid of tobacco mosaic virus very rapidly and at a rate appreciably faster than formaldehyde indicating their great reactivity towards nucleic acid Intact virus was inactivated much more slowly and only at very much higher concentrations Since many substances which react with proteins do not inactivate viruses and since most of the virucidal substances inactivate the free nucleic acid more rapidly than intact virus (Table III), it seems to be a general phenomenon that in order to inactivate a virus, a compound has to react with its nucleic acid Formaldehyde, which is widely used in the preparation of vaccines, also reacts readily with virus nucleic acid and leads very rapidly to inactivation (FRAENKEL CONRAT, 1954, STAEHELIN, 1958) It is not, however, a specific nucleic acid reagent since it has long been known to react with proteins Formaldehyde, being a very small molecule, can probably penetrate much more easily to the nucleic acid which in the virus is surrounded by a protein coat, than the much larger molecule, Kethoxal. The latter, though much more reactive towards the free nucleic acid, would be expected to inactivate the virus at a slower rate because of steric inhibition by the protein coat This is in accord with the experimental findings (Table III)

Porphyrins The haemagglutination of influenza PR 8 virus is inhibited after incubation of the virus with various porphyrins like Deuteroporphyrin and Chlorophyllin (KRADOLFER ET AL, 1957b) The inhibition occurs only in the presence of light, no effect being observed upon incubation in the dark The porphyrins, especially Deuteroporphyrin, were also found to reduce the infectivity of influenza A and B, Newcastle disease and ectromelia viruses but not of Columbia SK virus With the PR 8 strain of influenza, it was found in addition that upon short incubation with Deuteroporphyrin, the antigenicity of the virus was markedly reduced

Naphthoquinoniminines JUNGEBLUT (1951) in confirmation of experiments by SCHNITZER ET AL. has reported that the mortalities of mice after intraperitoneal infection with Columbia SK virus could be greatly reduced by the chemoprophylactic administration of a 2-hydroxy 1,4 naphthoquinonimine Against EMC virus only insignificant effects were observed Since the effect depended critically upon a quantitative relationship between virus and drug and since the virus was also inactivated in vitro, it was concluded that it was rendered non infective through a direct interaction between virus and drug

Polyelectrolytes L-Polylysine peptides have been demonstrated to

Table III Inactivation of Virus and Free Nucleic Acid

Inhibitor	Concentration	Time	Activity % of control	
			Virus	Nucleic Acid
Kethoxal	025 mg/ml	½ hour	100	81
	05 mg/ml	½ hour	100	23
	1 mg/ml	½ hour	100	6
	20 mg/ml	1 hour	100	0
	20 mg/ml	24 hours	57	0
Formaldehyde	25 mg/ml	½ hour	100	54
	5 mg/ml	½ hour	100	4
	10 mg/ml	2 hours	81	0
	10 mg/ml	24 hours	9	0
Chlorophyllin	01 mg/ml	2 hours	98	91
	1 mg/ml	2 hours	51	38
Deuteroporphyrin	01 mg/ml	2 hours	85	48
	1 mg/ml	2 hours	77	25
Ricin	01 mg/ml	2 hours	70	26
	1 mg/ml	2 hours	68	0

10 mg/ml of tobacco mosaic virus and 0 5 mg/ml of free nucleic acid were incubated at room temperature in 0 001 M Phosphate pH 6 8 during the time and with the concentrations of inhibitors indicated To remove free inhibitor before assaying samples containing virus were spun down twice in the ultracentrifuge and nucleic acids were precipitated four times with 2 volumes of alcohol in the cold Assays were conducted according to FRAENKEL CONRAT ET AL (1957)

inhibit the growth of influenza virus in the chick embryo (RUBINI ET AL, 1951) and to protect embryonated eggs infected with several strains of infectious bronchitis and Newcastle disease virus (GREEN ET AL, 1953) In experiments with mumps, a single injection of 0 2 mg of L-polylysine into the allantoic sac even when given 36 hours after infection reduced the haemagglutinin titer markedly When given into the yolk sac, a polypeptide of 4900 molecular weight was found

to be effective, while one of 20 000 molecular weight showed much less activity Since polylysine reacts with tobacco mosaic virus, proteins and nucleic acids, it was suggested that polylysine might act intracellularly by diffusing into the cell and combining with intact virus, virus fragments, or intermediates in virus formation (GREEN ET AL, 1953b) D Polylysine was found to be equally effective but ten times more toxic (TSUYAKI ET AL, 1956) GREEN ET AL (1954) found polyvinylamine also to be active A basic poly electrolyte rather than a peptide seemed to be the requisite for activity

Not only cationic, but also anionic polyelectrolytes inhibit the growth of myxoviruses NEHER ET AL. (1955) demonstrated the activity of several aromatic polysulfonic acids on the multiplication of influenza and Newcastle disease viruses in chick embryos and deembryonated eggs While being virucidal at higher concentrations, these compounds also appeared to affect the surface of red blood cells since they inhibited the virus haemagglutination as well as the iso haemagglutination of human red blood cells

Various anionic polymers have also been compared as to their ability to inactivate enzymes and viruses (HEYMANN ET AL, 1958) It appeared that the antiviral action was an expression of a general reactivity towards proteins rather than a specific reaction with viruses

Mucoproteins and Mucopolysaccharides

ALLEN ET AL (1958) have summarized the viral inhibitors which are present in normal animal sera and classified them into three groups (a) inhibitors of lipid nature extractable with fat solvents, (b) proteins protein complexes and mucopolysaccharides which are relatively stable to heat, and (c) heat labile proteins and protein complexes The mechanism of the second group seems to be best understood Mucopolysaccharides and mucoproteins from many sources have been shown to inhibit the haemagglutination reaction of the influenza group of viruses Many of them act by direct reaction with the virus (BURNET, 1951) The effect is due to their similarity with the receptor substance of the red blood cells and their affinity for the virus enzyme The action is seen in substances which are slowly attacked by the enzyme, thus having an affinity for the enzyme while at the same time blocking the sites necessary for adsorption It is seen even more markedly when the virus is slightly altered by

heating so that the enzymatic action, which leads to inactivation of the inhibitor, is more impaired than the affinity The functional unit in *all* these substances seems to be N Acetylneuraminic acid, since this substance is released by the virus and since, after its release, the inhibiting power on haemagglutination is lost (GOTTSCHALK, 1956)

N-Acetylneuraminic acid is also released by the receptor destroying enzyme of *Vibrio cholerae* (GOTTSCHALK 1958) Intraperitoneal injection of RDE resulted in a chemoprophylactic protection against infection with Columbia SK virus by the same route (VERLINDE AND DE BAAN 1949, JUNGEBLUT 1950) The presence of N-Acetylneuraminic acid at the sites of adsorption seems to be of importance, therefore, for the mechanism of the first peripheral infection

Miscellaneous Compounds

CNS depressant drugs Mice infected with Newcastle disease virus die of pulmonary consolidation Mice treated with Chlorpromazine HCl or Tetraethylammonium bromide, however, survived and showed no signs of pulmonary consolidation (OGASAWARA ET AL, 1958) This suggests the participation of the autonomous nervous system in the pathogenesis of pulmonary consolidation

Various compounds of which Apresoline (l Hydrazine phthalazine) was the most effective have also been shown to inhibit the propagation of Theiler's GD VII virus of mouse encephalitis in tissue cultures of mouse brain (PEARSON ET AL, 1955)

Phenolic compounds KRAMER ET AL (1955) found some phenolic compounds active against poliomyelitis virus in monkey testicular cultures Amino acids containing *sulfhydryl or hydroxyl groups* inhibited the action of the drugs HOLLINSHEAD ET AL (1956) found a series of similar compounds, mostly quinones, also to be active in the same system Type 1 poliomyelitis virus was more susceptible to some hydroquinones than was type 2 or 3 KNOX ET AL. (1957) have pointed out the danger, however, of using the cytopathogenic changes caused by the virus as criteria of virus growth with these compounds Some of them, like gallic acid, can "fix" the cells so that they look normal GEBHARDT ET AL (1955) have observed chemoprophylactic protection in monkeys against poliomyelitis virus upon introducing the maximum tolerated dose of some naphtholic and phenolic compounds into the stomach of monkeys 24 hours before

infection by the same route The most active compounds, especially the mother liquor of gallic acid, seemed to have no virucidal effects

Amethopterin This folic acid antagonist (HAAS AND STEWART, 1956), as well as folic acid deficiency (HAAS ET AL, 1957) prolonged the life of mice infected intracerebrally with lymphocytic choriomeningitis virus of mice The treatment affected the response of the tissue in a similar way as did x ray irradiation and resulted in a less intensive cellular reaction (LERNER AND HAAS, 1958) Interestingly, it does not prevent virus multiplication Actually, in mice which were infected subcutaneously, virus is recoverable from the blood and the pools of certain abdominal organs for several weeks in treated mice but only briefly or not at all in untreated mice

HURST (1957) has pointed out that dietary deficiency as well as toxic drugs can bring the animal into a sickly condition in which the reaction of the organism to the infection is altered and might appear less severe The finding that all of the treated animals showed evidence of toxicity before illness from virus had time to develop (HAAS AND STEWART, 1956) suggests that the general toxicity of the drug is responsible for the less severe reaction toward virus infection rather than a specific effect on virus synthesis

4 (3 hemisuccinyl deoxycholylamino) 4' hemisuccinylamino diphenyl sulfone This compound, which is a combination of a diphenyl sulfone and a steroid was reported by BERCZELLER (1959) to inhibit the PR 8 strain of influenza virus in chick embryos

Conclusions and Summary

From the many studies on antivirus drugs, the following conclusions seem to have evolved

1 Most compounds act only or at least best during the very early stages of infection, little activity being found once the disease has developed Their action therefore is chemoprophylactic rather than chemotherapeutic. This would not render them useless as far as clinical application is concerned, however, since a safe chemoprophylactic agent would be invaluable for instance during the outbreak of an epidemic

2 The majority of drugs are active in near toxic doses without allowing a wide enough range between toxic and inhibitory concentra

tion They do therefore not satisfy the requirements of a drug suitable for clinical application

3 Many agents, e g bacterial polysaccharides, thiosemicarba zones, mepacrine, etc are active only against certain viruses and not against others This could indicate that instead of one general antivirus drug, there might be a requirement for different drugs for various groups of viruses

4 The same virus may also be susceptible *to a drug only in a* certain animal species and not in another This is the case, for instance, with mepacrine and isatinthiosemicarbazone A study of the range of susceptible animals is therefore indicated with any compound which shows *activity in animals*

5 Another dependence on factors concerning the host is exhibited by differences in the activity of the drug according to which route is chosen for infection Examples of this are seen with Xerosine and 2,6 Diaminopurine

6 In order to have a protective effect in animals, it is not neces sary for a compound to inhibit virus synthesis completely Reduction of the infectivity titer in the affected organ by about one log unit is already sufficient to prevent the fatal outcome of the disease and might even be beneficial in as much as it leads to immunity in a way similar to that of attenuated viruses

7 Combined treatment with two substances of different modes of action has proven in certain cases to be superior to treatment with either substance alone Synergic effects have been found with two chemicals, isatinthiosemicarbazone and Dichlorophenoxythiouracil *or with antiserum and* a virus inhibitor, e g Myxoviromycin Helenine and Caprochlorone

8 There are indications that a drug can act, also, not by inhibit ing virus multiplication but by preventing the response of the tissue instead, which in certain cases determines *the fatal outcome of* a disease Xerosine and CNS depressant drugs are considered to act by this mechanism

9 One of the desired properties of an antivirus drug is that it be highly specific in interfering with virus synthesis while leaving the cell as little affected as possible One step in this direction is indicated by the effectiveness of β D ribofuranosides of pyrimidines and halogenated benzimidazoles which affect RNA and not DNA synthesis β Ribofuranosides were not only better inhibitors of virus growth than

free bases but, at least in one instance they were also found to be relatively less toxic

10 Assays in tissue culture can lead to quite different results than are found in animals Not only are many substances found active in tissue culture and not in animals but the reverse is also true Cinchoninthiosemicarbazone, e g is a weak inhibitor in tissue culture but one of the most potent in animals, while the opposite is true for pyruvic acid thiosemicarbazone (MINTON, 1953)

In summary, none of the compounds studied so far, which inhibit virus multiplication, satisfies the requirement for clinical application i e a combination of antivirus activity and low toxicity Promising leads, however, have been obtained along several lines

(a) Specific inhibitors of ribonucleic acid have generally been found to show high activity and are, as far as biochemical pathways are concerned, the most selective inhibitors of virus synthesis

(b) The systematic search for antivirus agents on an empirical basis has produced some synthetic chemicals and a number of antibiotics capable of inhibiting virus growth not only in tissue culture and eggs but also in infected animals

(c) Several compounds have been found which favorably influence the course of a virus disease not by inhibiting virus multiplication but by their action on the response of the host tissues

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Electron Microscopy of Viruses in Thin Sections of Cells Grown in Culture

By N. HIGASHI

Recent advances in tissue culture techniques, particularly refinements of cell culture procedures for the study of animal viruses by DULBECCO AND VOGT (1954) to provide an accurate assay method with poliomyelitis virus have made the study of kinetics of virus growth within infected cells far more easy and fruitful. On the other hand, in recent years the progresses in electron microscopy and ultra microtomy have been such as to allow us to obtain rather exact information concerning the intracellular events following viral infection and to get a general concept of structural development of some of the larger or intermediate sized viruses within host cells.

Following the pioneering work of REISSIG AND MELNICK (1955) who studied the multiplication of herpes B virus in thin sections of monkey kidney cells grown in culture, and the work of MORGAN ET AL. (1956) who studied the adenovirus in thin sections of infected HeLa cells, considerable success has been attained in recent years in the clear demonstration of multiplication of some viruses within

host cells. High resolution electron microscopy using ultrathin sectioning is unique in furnishing molecular or supra molecular morphological information about virus multiplication by direct electron optical imagery. Most of experimental and observational materials presented here are drawn from works with which the author has been closely associated.

Electron Microscopy of Pox Viruses in Thin Sections of Cultured Cells

A contribution of electron microscopy to the deep understanding of the most fundamental intracellular events following viral infection might be made by studying cells in tissue culture infected with virus, in which a relationship could be established among the growth cycle of virus determined by infectivity titrations, the cellular alterations under the conventional light microscope and the presence of virus or materials or components associated with virus within the cells seen in the electron microscope.

Ideal electron microscopy of viruses in thin sections requires that a virus host cell system can be utilized in which all the cells are equally susceptible to the virus and in which all can be infected within a brief period simultaneously, in order to insure that the samples are truly representative of infected cells taken at different stages of infection. Electron microscopists may consider that the total *areas of cells examined are extremely small.*

Before describing the electron microscopy of pox viruses in thin sections, growth cycle experiments and conventional light micro scopy of these viruses will be given.

Growth Cycle Experiments

The results of growth cycle experiments will be shown in the ectromelia virus L cell system (Fig. 1) and the variola virus HeLa cell system (Fig. 2). In Experiment *I* in the former, 5×10^5 cells per ml received virus inocula of $10^{6.2}$ $TCID_{50}$ per ml and in Experiment II, 7×10^5 cells, $10^{6.35}$ $TCID_{50}$ respectively. In the latter, 5×10^5 cells per ml received virus inocula of $10^{6.55}$ $TCID_{50}$ per ml. In these experiments no increase in the amount of cell associated virus was observed during

the first 4 or 8 hours (time zero means the time when cells were inoculated with virus) Subsequently, a steady increase in virus titer was noted and a peak virus titer was attained in 15 and 18 hours, respectively The rises in the amount of cell associated virus in such

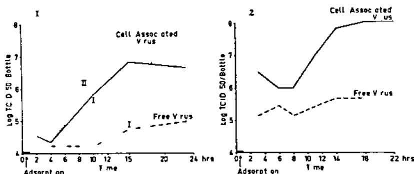


Fig 1 Growth curves of HAMPSTEAD strain of ectromelia virus after inoculation of the cultures with the same inoculum of 3 2 TCID$_{50}$per cell in Experiment I and II *Fig 2* Growth curve of YAMAMOTO strain of variola virus after inoculation of the cultures with 7 1 TCID$_{50}$per cell

growth cycle experiments were about 300 to 500 fold for ectromelia virus and about 100 fold for variola virus (HIGASHI AND OZAKI, 1957, ICHIMIYA, 1959)

The curves for free virus in the experiments showed that increase in free virus was less rapid than increase in cell associated virus For example, in Experiment I increase in free virus was not detected until at least 4 hours after cell associated virus had begun to increase

Conventional Optical Microscopy

The following was the typical sequence of events following inoculation of 4 to 5 day culture of strain L cells or HeLa cells (10^6 cells per ml) with $10^{6.1-6.11}$ TCID$_{50}$ per ml of ectromelia, vaccinia or variola virus, which were parallel studies of the growth cycle experiments described above No marked differences between cells in infected tubes and cells in control tubes were observed during the first 3 hour period At the fourth hour, small, often multiple circular or oval basophilic inclusion bodies (stained blue by hematoxylin and eosin

after ZENKER fixation) surrounded by a clear zone were first observed in the cytoplasm of a small portion of the infected cells, but were more striking in the subsequent stages as shown in Fig 3 to 5, taken at the sixth, eighth and twenty fourth hours respectively In some preparations virtually every cell was found to contain at least one inclusion At the later stage inclusions tended generally to be of larger size, less regular in outline Finally, in the case of the variola virus HeLa cell system, inclusions tended to be less basophilic and dispersed into the cytoplasm FEULGEN reaction of the basophilic inclusions was positive in all stages

In the case of the vaccinia virus HeLa cell system and the variola virus HeLa cell system, eosinophilic inclusions were never detected at any stage of infection However, in the case of the ectromelia virus L cell system the eosinophilic round or oval intracytoplasmic inclusions began to be observed approximately at the tenth hour, subsequently these inclusions increased in size and number, the shape remaining almost the same, never dispersing into the cytoplasm Therefore, cells beyond the tenth hour were found to contain both types of inclusions in the same cell

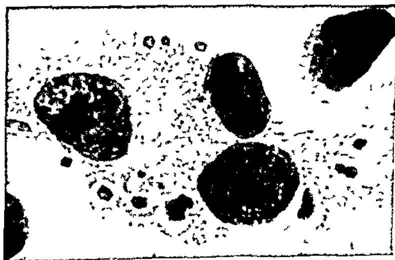


Fig 3 Strain L cell culture 6 hours after inoculation with the HAMPSTEAD strain of ectromelia virus Basophilic cytoplasmic inclusion bodies surrounded by a clear halo are present in the cell Fixed in ZENKER's fluid and stained with hematoxylin and eosin Magnification 1700 × (HIGASHI AND OZAKI, 1958)



Fig. 4. A more advanced stage of the infection (8 hours after inoculation) than that in Figure 3. Every cell contains basophilic inclusions. ZENKER's fixative. Hematoxylin and eosin stain. 1000×. (H CAS I AND OZAKI 1958)



Fig. 5. HeLa cell culture 24 hours after inoculation with the YAMAMOTO strain of vaccinia virus. Basophilic cytoplasmic inclusion bodies in irregular shape are seen in the multinucleated cell. ZENKER fixation, stained with hematoxylin and eosin. 1500×. (ICHIMIYA 1959)

Electron Microscopy

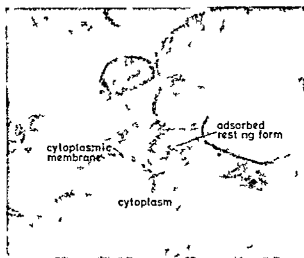
At the same time that the growth cycle experiments and optical microscopies were carried out a parallel series of cultures was examined for electron microscopy Studies of pox viruses in thin sections will be mentioned as follows

virus to cell adsorption
formation of inclusions
viral development in an early stage of infection
formation of mature viral particles
discussion

Virus to Cell Adsorption

The electron microscope has been applied to the visualization of the adsorption that takes place between red blood cells and the myxo viruses (DAWSON AND ELFORD 1949) ANDERSON (1952, 1953) has investigated by electron microscopy the nature of adsorption of bacteriophage particles on host bacteria revealing that the primary stage of phage adsorption is by the tips of their tails Recently, interesting electron micrographs of hemadsorption in cultured monkey kidney cells infected with influenza virus were obtained by HOTCHIN ET AL (1958), who found that the phenomena occurred by two different processes In one, the red cells were attached to virus fila ments protruding from the host cell separating the two cells by some distance (virus hemadsorption) In the other, the binding between the two kinds of cells was direct and tight with no intermediary virus (cyto hemadsorption) These two forms of attachment can be distin guished only by electron microscopy In addition, the electron micro graphs suggested that there occurred an apparent change in the immunologically specific structure of the host cell surface following infection with influenza virus

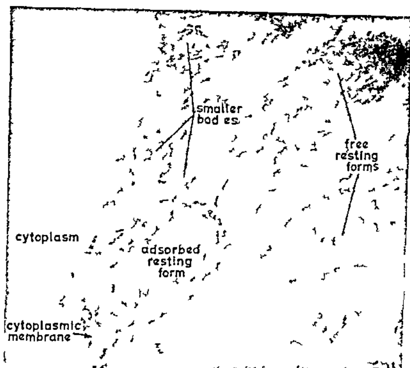
ADAMS AND PRINCE (1957) showed the electron micrographs of particles adsorbed on the EHRLICH ascites tumor cells which they tentatively identified as newcastle disease virus Similar particles occasionally might be encountered and confused with sections of cell components involved in specimen preparation of free cells having many pseudopodia It is not hard to take such photographs as those reported by them, since unfortunately cross sections of cell components



F g 6 Electron m crograph of stra n L cell wh ch had been bathed in a flu d of ectromel a v rus for 1 hour at 37° C to allow adsorption of v rus to the L cells Character st c rest ng form of the v rus 200 mμ in ma or ax s is adsorbed on the depressed cell surface Magn fcat on 90 000× (H GASH AND OZAKI 1959)

like those of newcastle disease virus are often photographed attached to the cell surface

No electron microscopic information is at hand regarding the entrance of animal virus into the host cell No experiments have yet been reported of any electron microscopy of attachment of pox viruses to host cells prior to infection Recently ectromelia or variola virus adsorbed on the strain L cells or HeLa cells were photographed using ultrathinsectioning in our laboratory (HIGASHI OZAKI 1959 ICHIMIYA 1959) The particles seemed to touch tightly the host cell cytoplasmic membrane orienting often obliquely with its major axis towards the cytoplasmic membrane (Figs 6 and 7) The particles are easily identified as resting forms of ectromelia or variola virus The following supports this identification Their measurements agree with estimates of the size of resting form of these viruses They have the same unique complex structures as the resting forms of these viruses Similar particles have never been seen on the thin sections of control strain L cells or HeLa cells In some sections the outer double membrane of the viral particle and host cell cytoplasmic membrane were separated by a distance of up to 200 Å (Fig 7) In many instances viral particles were crowded around the cell surface and usually the portion of the

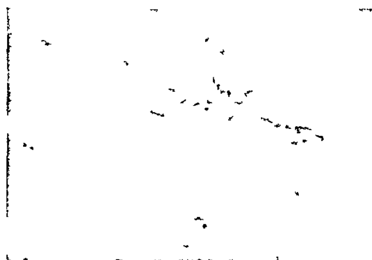


F g 7 Rest ng form of ectromel a v rus adsorbed on the surface of s ra n L cell s present at lower lef Note he dep ess on of the cell surface where v ral par cle adsorpt on is seen Ano her st k ng fea ure s nterm ngl ng of three sma er bod es h ral part cles The bod e smaller than nucleo d are repea edly encoun ered n adsorp on expe men s
180 000 × (H GAS AND OZAK 959)

host cell membrane where viral particle adsorption was seen ap peared to coincide with a depression of the cell surface The significance of occasional occurrence of empty viral particles attaching to the host cell surface is not known although this phenomenon recalls the findings in bacteriophage studies (HERSHEY AND CHASE 1952 ANDERSON 1953)

Formation of Incl sions

Intracytoplasmic inclusions appeared 3 hours following infection in all three infectious systems Thereafter they increased rapidly in size and numbers The features of the inclusions appeared to be related both to the fixatives used and to the developmental stage of



Fg 8 T o rest ng forms f ar la v rus a l rbed on the surface f HeLa cell is seen The part cles are cut h r z ntally and the cell surface is sl ghtl depressed 120 000 (Hic as i ani Ici ida 1959)

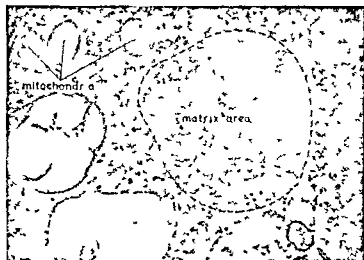


Fig 9 A p rt n f cyt plasm of stra n L cell 4 hours af er in culat on w th ectr mel a rus An ntracyt plasm c inclus n (matrix area) f xed with osm c ac d s l cated near the center B undary f the ma r x area s irregular and appears denser than the rest f cyt plasm Three m toch ndria are seen at upper left 35 000 (H c as i Ozak and Notake 1958)

the inclusions. In an early stage of their development, inclusions fixed with osmic acid showed fine reticular material of greater density than the rest of the cytoplasm. Their boundary was sharp but irregular as Fig. 9 taken at the fourth hour illustrates. In contrast to this, inclusions fixed with potassium permanganate (LUFT's method, 1956) had homogeneous structures showing lower density with no sharp boundary or irregular shape as Fig. 10 taken at the sixth hour demonstrates (HIGASHI, OZAKI AND NOTAKE, 1959).

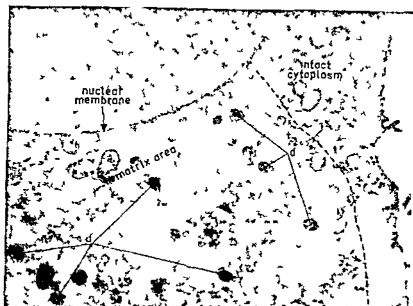


Fig. 10. Cytoplasmic inclusion (matrix area) formed 6 hours after inoculation with ectromelia virus. It occupies about four fifths of the cytoplasm. Fixation with potassium permanganate. Contrast of matrix area with the rest of cytoplasm is not sharp. Inclusion contains several dense developmental forms of the virus. Nucleus with double nuclear membrane is seen at upper left. 14 000 × (HIGASHI, NOTAKE AND OZAKI, 1959)

The inclusion material was altered in some way during the development of the inclusions, structures becoming more condensed at their centers or peripheries resulting in very irregular outline. Finally the material almost faded away, presumably being incorporated into the newly replicated virus particles.

At a later stage of infection, samples taken from the ectromelia L cell system presented another quite different intracytoplasmic inclu-

sion which had, in striking contrast to those just described, greater density with homogeneous mass and sharp boundary, circular or oval shape as Fig 11, taken at the thirty-second hour, illustrates (HIGASHI ET AL, 1957, 1958) With time, they increased in size and number, but their density and shape remained almost all the same, never dispersing into the cytoplasm Such inclusions were never observed at any stage of infection either in the vaccinia virus HeLa cell system or the variola virus HeLa cell system (ICHIMIYA 1959)

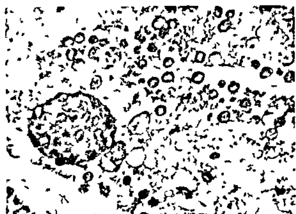


Fig 11 A classical inclusion body (Marchal body) formed in matrix area after 32 hour *infection is present at left It contains many viral particles Note the* sharp boundary higher density A large number of developmental forms are scattered throughout the matrix area Osmic acid fixation 14 000 × (HIGASHI ET AL, 1958)

The inclusions appearing first in thin sections, which are now generally called ' matrix" area according to the original description by MELNICK (1953), can be identified as the basophilic inclusions appearing first in the light microscope The second inclusions will be identified as classical eosinophilic inclusions (Marchal body) This identification will be supported by the following The time sequence of appearance of the first inclusions (matrix area) and the basophilic inclusions is similar in electron microscope findings and in optical findings The progressive structural changes of the matrix area and those of the basophilic inclusions are quite similar The second inclusions were observed only in the ectromelia virus L cell system at a later stage of infection in the electron microscope Corresponding to this,

in the light microscope approximately at the same stage of infection the Marchal bodies began to be detected in the same system An incrase in size and number, and the other features of the second inclusions noted in the electron micrographs generally parallel similar changes in the Marchal bodies seen with the light microscope.

Viral Development in an Early Stage of Infection

The first recognizable viral forms were the so-called developmental forms, which were elliptical, circular, or hemicircular in profile and had an average diameter of approximately 270 mμ in round form They were exclusively observed within the matrix area in an early stage of infection (6 hour infection), never detected in the classical inclusions which will be formed in a later stage Forms composed of "nucleoid" (MORGAN ET AL., 1954) separated from the viroplasm by a narrow zone of lesser electron density (Figs 12 and 13), other forms with an area of lesser density filling a variable extent of the viroplasm (Fig 14)



Fig 12 A portion of strain L cell culture 8 hours after inoculation with ectromelia virus Matrix area containing several kinds of developmental forms is seen at the central area of cytoplasm Density of matrix material is low Osmic acid fixation 16,000 × (HIGASHI ET AL., 1958)



Fig. 13. Developmental forms of ectromelia virus formed in matrix area of 6 hour infection in L cells. Limiting membrane of the right developmental form is about half completed but clearly double in the remainder of the form. It is hard to see membrane in the other two forms. Nucleoid about 1 0 80 mμ can be seen at lower left one. × 90 000 (Higashi and Ozaki 1959)

and the other several kinds of developmental forms including forms having no nucleoid (Figs 15 and 16) were repeatedly encountered in the sections of 6 hour infection in all three infectious systems examined. At this stage the matrix areas never contained mature forms which will be mentioned later. In a later stage developmental forms and mature forms appeared in the same matrix areas.

Most of the developmental forms were limited by a double membrane system as an outer dense line measuring 40 to 50 Å in width and an inner dense line measuring almost the same width (Higashi and Ozaki 1959). The details of the membrane structures differed from one type to another. Forms without complete membranes were sometimes noted (Fig. 16). It is very interesting to note that structural appearance of the viroplasm was almost the

same as that of the dense material in a matrix area as illustrated by Fig 16, and that coiled threads measuring about 30 to 50 Å in width were seen in viroplasm (Figs 14 and 15) In Fig 16 viroplasm was continuous with the matrix material without completion of its circle, showing just the same appearances in density and structures

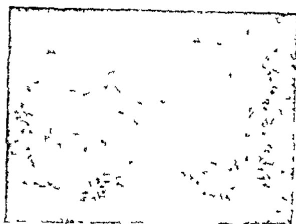
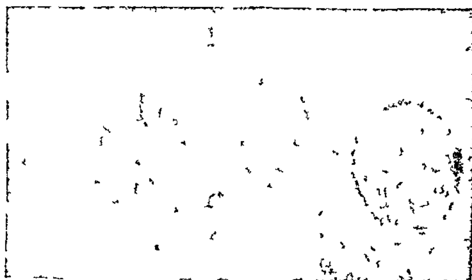


Fig 14 Two developmental forms 280 × 220 mμ from the same culture as in Fig 13 showing lesser density area in which coiled thread like structures are present The two irregular ellipsoids point the same direction due to deformation by cutting 150 000 × (HIGASHI AND OZAKI 1959)

Formation of Mature Viral Particle

Fig 17 shows mature forms of variola virus which were observed in the matrix area in a later stage of infection (22 hour infection) Fig 18 shows mature forms within MARCHAL body Also in Fig 6 to 8 mature forms can be seen The structures of the mature forms show quite characteristic appearances and are easy to distinguish from the developmental forms by the presence of two kinds of double membranes, by their shape, by smaller size (about 240 × 200 mμ with considerable individual variation) and by rather stronger density in thicker sections One of the double membranes measuring 30 to 40 Å in width encloses the internal body, i e, nucleoid This nucleoid membrane resembled that observed in other experiments (MORGAN ET AL, 1954) The other was a double membrane enclosing viroplasm which had a broader space between two lines than that of the developmental



F g O he de el pmen a fo ms h h ha e c ea double memb anes excep ex emely lef one \ op asm shows abou he same g de of c mp ex s ruc u es comp ed of h ead measu ng ab u 30 o \ n w d h 30 000 (H GA AN OZAK 959



F g 6 Th m c g aph shows ha he opl m f de e pmen al f rm s c n nu us w h ma x ma c ial 80 000 (H s D O AK 959)

same as that of the dense material in a matrix area as illustrated by Fig 16 and that coiled threads measuring about 30 to 50 Å in width were seen in viroplasm (Figs 14 and 15) In Fig 16 viroplasm was continuous with the matrix material without completion of its circle showing just the same appearances in density and structures

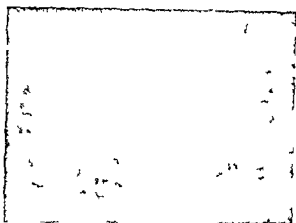


Fig 14 Two developmental forms 280 × 220 mµ from the same cul ure as n Fig 13 show ng lesser dens ty area n wh ch co led thread l ke s ruc ures a e present The two rregular ell pso ds po nt the same d rect on due to deformat on by cutt ng 150 000 × (H CAS AND OZAKI 959)

Form ation of Mat re Viral Particle

Fig 17 shows mature forms of variola virus which were observed in the matrix area in a later stage of infect on (22 hour infection) Fig 18 shows mature forms within MARCHAL body Also in Fig 6 to 8 mature forms can be seen The structures of the mature forms show quite characteristic appearances and are easy to distinguish from the developmental forms by the presence of two kinds of double membranes by their shape by smaller size (about 240 × 200 mµ with considerable individual variation) and by rather stronger dens ty in thicker sections One of the double membranes measuring 30 to 40 Å in width encloses the internal body i e nucleoid This nucleoid membrane resembled that observed in other experiments (MORGAN ET AL 1954) The other was a double membrane enclosing viroplasm which had a broa ler space between two lines than that of the developmental

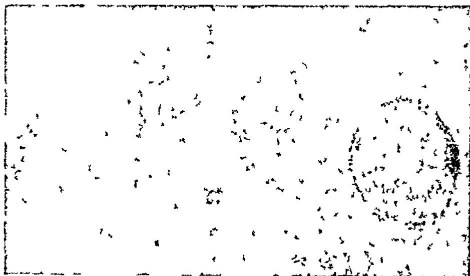


Fig 15 Other developmental forms wh ch have clearly double membranes except extremely left one V roplasm shows about the same grade f complex structures composed of threads measuring about 30–50 Å in width 130 000 × (HIGASHI AND OZAKI 1959)

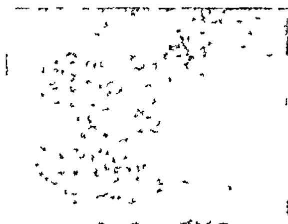


Fig 16 Th s m crograph sh ws that the viroplasm of developmental form is continuous with matrix mater al 180 000 × (HIGASHI AND OZAKI 1959)

form It is interesting to note that the structures resembled those observed in thin sections of viruses of variola and vaccinia by ANDRES ET AL (1958)

As mature forms began to appear the density of matrix areas decreased gradually When the areas were occupied by a large number of mature forms as well as developmental forms they were often characterized by disappearance of the matrix material

In the case of the ectromelia virus L cell system classical inclusions containing mature forms appeared in or near the matrix areas which were occupied by mature forms and/or developmental forms Observations of the ectromelia virus Yoshida sarcoma cell system also followed almost the same pattern (Fig 19 OZAKI 1956) However in the other two systems examined the formation of classical inclusions never occurred



Fig 17 Resting forms of variola virus within matrix area 210 000 × (HIGASHI AND OZAKI 1959)

Discussion

On the basis of evidence obtained so far the development of pox viruses (variola virus vaccinia virus and ectromelia virus) can be outlined as follows The initial sites of development are confined to a special area of the host cell cytoplasm At the very early stage of in

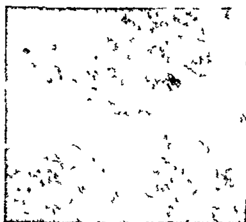


Fig. 18 A portion of MARCHAL body in which characteristic resting forms are visible. 150 000 (HIGASHI AND OZAKI 1959)

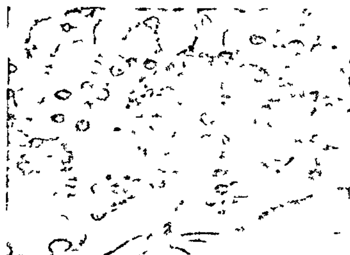


Fig. 19 Cytoplasm of YOSHIDA sarcoma cell infected with ectromelia virus. There are two MARCHAL bodies which contain a number of resting forms of the virus. Note that developmental forms are scattered throughout the cytoplasm probably matrix area of cytoplasm and compare it with MARCHAL body in Fig. 11. 32 000 × (OZAKI 1956)

fection (3-hour infection) at one or more foci an accumulation or a production of unusual material showing high or moderate electron density replaces or displaces the cytoplasm of the infected host cell The focus constitutes the matrix area Within the matrix all the necessary components for virus fabrication are produced and accumulated in increasing amount An area containing such material is invariably *associated with infected cells* At the fourth to fifth hour structures of coiled thread often can be seen in the matrix material Structures could *not* be *associated with the gross morphology* of viral particle

At a little later stage viral forms appear *in the matrix areas* (6 hour infection) Some workers suggested that the membranes seemed to form independently (MORGAN ET AL, 1954, TAJIMA AND KUBOTA, 1957) Such interpretations of the process may not be correct It seems that the membrane itself does not emerge independently either before or after the formation of viroplasm, but the membrane will differentiate from the original sphere or incomplete sphere which represents the first recognizable viral developmental form

MORGAN ET AL (1954) indicated that developmental forms were enclosed by a single membrane in the case of viruses of vaccinia and fowl pox developed on chicken embryo chorioallantoic membrane *and a double limiting membrane appeared after the formation of the* complete viral particle These earlier findings can now be amplified, and from the observations described here it is possible to offer a fairly precise morphologic description of these pox viruses Most of these developmental forms definitely have a double limiting membrane irrespective of fixative (OsO_4 or $KMnO_4$) as Figs 13 to 16 illustrate

The nucleoid appears quite dense in thicker sections (Fig 12), but it should be noted that coiled thread like structures occupy the nucleoid area in very thin sections This, for example, recalls the observations that the cell nucleolus having no structure in thicker sections comes to show some structures in thinner sections In spite of intensive electron *microscopy of the matrix areas of this stage of* infection, we failed to confirm the findings of MORGAN ET AL (1954) that "*very dense material* appears within the matrix area and fragments into small units", which are incorporated into the virus with the viroplasmic component to constitute the nucleoids

In general, the membranes fixed with OsO_4 appeared more opaque than those fixed with $KMnO_4$ The appearance of the membrane showing osmophilia might be an indication of the presence of protein,

and the area of nucleoid might indicate the presence of DNA molecules, although at the present time it is indistinguishable in a section between a material with the mean density of protein and the mean density of nucleic acid Differential electron staining methods should be pursued Here the author tentatively proposes that structures in the nucleoid revealed under the high resolution electron microscope may present an aggregation of DNA molecules or nucleoprotein, i e aggregates of chromonema The experiments on the treatment with DNase, RNase and so on are now under way

At the present time it is very hard to arrange an experiment along a time sequence of the transitional developing forms described above, probably because the forms apparently undergo a complex life cycle through a transitional stage and the maturation time from one form to another cannot be detected by the present technique and so it is nothing but speculation if one interprets their time sequence

The developmental forms are elliptical or circular in profile It is probable that the ellipticity noted is an artefact caused by compression during the cutting procedure since the major axis is invariably oriented perpendicularly to the direction of the ultra microtome glass knife, and since the ellipsoids photographed always point the same direction in the same field, not at random (see Fig 14) Therefore, probably the true silhouette of the developing forms is more nearly circular Electron microscopy of purified samples of infected cells which had exclusively contained developing forms showed quadrangular shape, due to the conventional preparation procedures (HIGASHI ET AL, 1958)

The final stage is the appearance of the particles smaller than the developmental forms The particles no longer present the characteristic features of developing form The structures of the final form of viral development, the so called "mature form" are characterized by an internal body with a double limiting membrane separated from the double limiting membrane of viroplasm The internal body appears essentially to be a disk which shows variable shape and density corresponding to cutting orientation (MORGAN ET AL, 1954, ANDRES ET AL, 1958, HIGASHI ET AL, 1959) These features are essential and fundamental differences between developmental forms and mature forms Mature forms appear to complete their maturation within the matrix areas and stay there until migration to other places in cytoplasm and release into the extracellular space When the maturation process was completed, matrix materials were almost exhausted and faded away The

transition from developmental forms to mature forms has not been studied in detail

Now let us discuss first the developmental form in relation to infectivity, and second the significance of formation of classical inclusion bodies First, as the growth curves with pox viruses in the strain L cells or HeLa cells show, the virus titer begins to increase in cell extracts after a constant period (6 hours) and a maximum titer is reached about 15 to 17 hours after infection Thin sections taken 6 to 8 hours after infection exclusively reveal several kinds of developmental forms, and mature forms and classical inclusions are never detectable Classical inclusions are undetectable also under the optical microscope These findings show that only developmental forms are responsible for infectivity in this stage The name of "developmental form" may have been defined only from the morphogenesis point of view Now it will be concluded that the developmental form named in the sense of morphogenesis is matured infective virus from the point of view of dynamic infectivity, and that the so called "mature form" appearing in the later stage is the "resting form" of virus

With the conventional light and phase microscope, KAMAHORA and his coworkers (1955, 1957, 1958) reported in detail the formation of two kinds of intracytoplasmic inclusion bodies in the same cell of the tissues infected with ectromelia fowl pox and vaccinia viruses Using the electron and phase microscopes, DOHI (1955) also described the same phenomenon in Ehrlich ascites tumour cells which had been inoculated with ectromelia virus and incubated in the peritoneal cavity of the mouse With the electron and light microscope HIGASHI ET AL (1958) observed the two kinds of cytoplasmic inclusions in the strain L cells infected with ectromelia virus FURUSAWA ET AL (1958) also found the same thing using the same infectious system with the light microscope

One of them is a classical eosinophilic inclusion body (Marchal body) which shows throughout the period of inclusion development a sharply demarcated ovoid or circle with the electron and light microscope The other is a basophilic inclusion body (matrix area in the electron microscope) which is oval or round during the initial period of inclusion development and then begins to show irregular shape In a striking contrast with the eosinophilic inclusion, with the electron microscope the basophilic inclusion shows different appearances depending on fixatives, and disperses into the cytoplasm of the

host cell in the latest stage of inclusion development In the HeLa cells inoculated with vaccinia virus or variola virus there forms only the basophilic type inclusion, which has quite the same features as described above In these two infectious systems classical eosinophilic inclusions (Guarnieri bodies) are never detected at any stage of infection with either the light or the electron microscope

The following facts should be stressed here Matrix areas (basophilic inclusions) are formed in the very early stage of infection, i e, in the eclipse period, and during the initial period of inclusion development nothing but matrix material can be demonstrated in the matrix areas Here virus formation proceeds in its early stage On the other hand, classical eosinophilic inclusions referred to as Marchal bodies are formed in a later stage after the virus titer has already increased near the peak and yet the inclusions always contain *mature viral particles* (which at the present time can be differentiated precisely from developmental form only by the electron microscopy), if they contain any viral forms It was reported that among the pox virus host combinations used, some combinations appeared to have showed the presence of classical inclusions containing virus, others appeared to have showed inclusions containing no virus, still others did both in the same preparations (Doi, 1955, Mizuguchi, 1955, Hagiwara, 1956, Wada, 1957) In addition classical inclusions were never observed in the HeLa cells infected with vaccinia or variola virus These viruses appeared to have developed entirely within the matrix areas (Higashi, 1958, 1959, Ichimiya, 1959) On the basis of these several evidences obtained, it will be reasonably concluded that intracytoplasmic classical eosinophilic inclusions of ectromelia virus may not be sites of virus replication, but they may be only depots of the viral particles which have initiated their development in the basophilic matrix areas and completed viral maturation at the expense of the matrix material The fact that in the HeLa cells inoculated with vaccinia or variola viruses no evidence of classical inclusion formation has been obtained seems to depend greatly on the severe damaging effects of the virus multiplication on the host cells, resulting in too quick appearance of cytopathogenic effects This recalls the *well known findings that in the liver cells of the mouse infected with* ectromelia virus Marchal bodies are rare

If one proposes that eosinophilic inclusions must be sites of virus multiplication, it should be demonstrated that the process of maturation of virus will take place within them Accumulation of mature

viral particles within the eosinophilic inclusions never means that they must be sites of virus formation In the light of the above, allow the author to criticize briefly some results reported so far GAYLORD AND MELNICK (1953) first successfully and beautifully demonstrated the processes of emergence of vaccinia virus with the electron microscope, which usually took place in matrix areas This is quite true, but their earlier working hypothesis that the processes also apply to the classical inclusions (Guarnieri body) may not be true In the case of fowl pox virus in cells of the chorioallantois EAVES AND FLEWETT (1955) called attention to the classical inclusions (Bollinger body) which they consider as sites of virus multiplication They may be mistaken, because they showed only accumulation of virus particles in the Bollinger body which were all surely mature forms, in addition, chorioallantois observations were made at a later stage

In summary, it seems to cover most of the facts to say that it is the basophilic inclusions which play a necessary role in supplying material for viral multiplication The eosinophilic inclusions, if produced, are only by products depending on a little understood host virus relationship in the rather late stage of infection The appearance of the basophilic inclusions are signs that virus multiplication is going on in those areas

Electron Microscopy of Viruses of the Psittacosis Lymphogranuloma Group in Thin Sections of Cultured Cells

Electron microscopy of the viruses of this group in thin sections of infected tissues has been made by GAYLORD (1954), TAJIMA ET AL (1957), and MITSUI ET AL (1958) So far as the author is aware, no such study using cells grown in culture has been reported Most of electron microscopic observations presented here will be drawn from work which has been done in the author s laboratory using cultured strain L cells (HIGASHI ET AL , 1958, 1959)

Virus used was the Cal 10 strain of meningopneumonitis virus which had been transferred many times in the yolk sac of embryonated eggs before being received by the author's laboratory The strain was subjected to 20 passages in strain L cells Strain L cells (4×10^{5} cells per ml) received virus inocula of $10^{7.1}$ MLD_{50}ml Although growth cycle experiments have not yet been completed, optical microscopy was done parallel with the electron microscopy

Conventional Optical Microscopy

Optical microscopic examinations according to MACCHIAVELLO's method were made on smears of cultures prepared during the various stage of incubation The cells after an adsorption period of two hours clearly showed the presence of many adsorbed particles, which stained red on a blue background The finding that the attached particles could be identified as elementary bodies of the virus examined is supported by the following evidence The staining reaction was quite similar to staining character of the elementary bodies of the virus (typical color reaction of the elementary bodies) The red particles were associated with infected cells and were not attached to cells from control tubes, and they were not observed extracellularly With time, the red particles migrate to the perinuclear areas (HIGASHI AND NOTAKE, 1959) This observations suggested that the infecting virus seemed to invade successfully a susceptible cell as such without breaking it up and generally conformed to the early descriptions given by BLAND AND CANTI (1935)

After 5 hour infection many red particles were crowded around the central region of most of the cells After 8 hour infection, particles which were stained blue began to appear with the progressive enlargement of the individual blue particle As infection proceeded larger blue particles of variable size were frequently intermingled with smaller red particles which gradually decreased in number In 12 hour infection the blue particles increased in number and cytoplasmic inclusions (matrices plaques, morulae) were often associated with the blue particles The inclusions grew in size and became ill defined, and it was hard to discern blue particles individually Subsequently newly formed red particles became to appear within the inclusions At the 27th hour red particles again became predominant and at the 32nd hour all particles recognized were red particles without detection of blue particles

Electron Microscopy of Meningopneumonitis Virus in Thin Sections of Cultured Cells

In an early stage of infection (12 hour infection) there appeared small inclusions measuring 1 0 to 1 5 μ in diameter which were extremely transparent to the electron beam in the specimens fixed with osmic acid Some of the inclusions contained a small number of large



Fig 20 Strain L cell culture 12 hours after inoculation with the Cal 10 strain of meningopneumonitis virus Note extremely transparent inclusion cavities which exclusively contain large bodies 9 000 × (HIGASHI AND NOTAKE 1959)

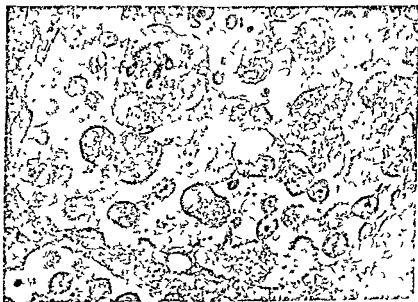


Fig 21 Inclusion in 18 hour infection with Cal 10 strain Pleomorphic large bodies are present 33 000 × (HIGASHI AND NOTAKE 1959)

bodies which could be essentially analogous to blue stained particles appearing at the same stage under the light microscope The shape of the large bodies was round to ovoid measuring 0 5 to 1 0 μ in diameter *Other bodies which will be mentioned later were not observed* in inclusions at this stage (Fig 20)

Subsequently the large bodies appeared pleomorphic, with irregular shape, huge form, dumbbell shape or forms beginning to segment They showed fibrous or reticulo granular structures surrounded by a single or double limiting membrane, and in some of them central or eccentric granules were visible (Fig 21) In 24 hour infection inclusions enlarged and were occupied by a large number of intermediate sized bodies and elementary bodies The large bodies greatly decreased in number and it was hard to detect their irregular shape (Fig 22) This stage of viral development corresponds to the stage



Fig 22 Inclusion of 24 hour infection with Cal 10 strain Several types of intermediate bodies elementary bodies and a few large types of large bodies are illustrated here There are dense solid bodies hollow bodies with a dense shell, and those which consist of a central or eccentric nucleoid within a thin membrane These appear to represent degrees of maturation Some of the membranes show microtome distortion and some are ruptured 12 000 × (Higashi and Notake, 1959)

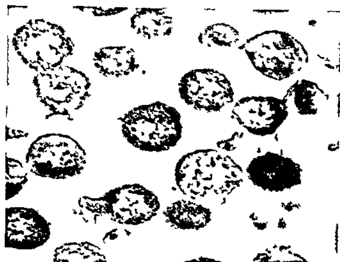


Fig 23 Another section from same culture as Fig 22 illustrating the virus at different stages of its development Intermediate bodies (400 430 ×) 330 mμ less dense in their center are present and some of them are about to divide 40 000 × (HIGASHI AND NOTAKE 1959)

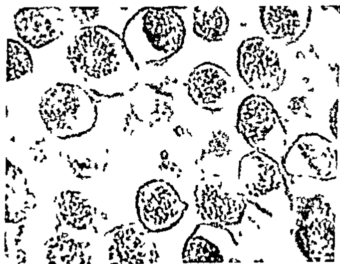


Fig 24 Intermediate sized bodies of meningopneumonitis virus All of them show characteristic coiled thread structure 45 000 × (HIGASHI AND NOTAKE 1959)

in which red particles are predominant as seen by the optical micro scope

Intermediate bodies were enclosed by a double membrane Of the intermediate bodies some appeared less dense in their center some were about to divide or appeared in pairs being either of equal or unequal size due to constriction or segmentation (Fig 23) and still others had a dense central or eccentrically located nucleoid which showed a coiled thread framework presumed to be aggregations of chromonema (Fig 24) The average diameter of the round form of intermediate bod es measured 400 m in diameter Elementary bodies about 250 to 300 m across were surrounded by a double membrane and had a nucleoid about 200 to 250 m in diameter showing stronger

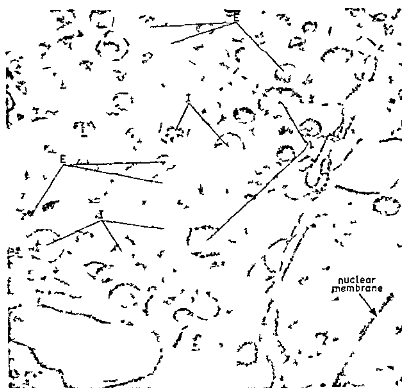


Fig 25 Sect on from 48 hour cul ure show ng elementary and n ermedia e bod es scattered throughout the cytoplasm A f w large bod es are also seen There s nuclear membrane at the r ght c rner 5 000 (H AS AND NOTAKE 959)



Fig 26 A "initial body inclusion" from trachoma at onset Large initial bodies and elementary bodies with dense or faint nucleoid are present 21,000 × (Courtesy of Y MITSUI 1958)

electron scattering power in thicker sections but not so in thinner sections (Fig 25) Transitional bodies from intermediate forms to elementary bodies were repeatedly observed characterized by decreasing size and structural differences of a central mass In a later stage these bodies were scattered singly throughout the cytoplasm of the host cell, still accompanied by a small number of large forms as illustrated by Fig 25 Fig 26 shows a trachoma inclusion containing large bodies and elementary bodies

Large bodies are entirely different from elementary bodies in electron optical morphology and optical staining reactions However, transitions from large bodies to final elementary bodies can be followed in thin sections with the electron microscope There is no doubt that large bodies are morphological precursors of elementary bodies The author is quite agreeable with electron microscopic observations on the viruses of the psittacosis-lymphogranuloma group (Maeda strain and Cal 10 strain) in mouse lung or in chorioallantois (TAJIMA ET AL , 1957) and studies on the trachoma inclusion from trachoma patients (MITSUI ET AL , 1958) in which the presence of intermediate forms of "initial bodies" (large bodies in this paper) in trachoma inclusions and elementary bodies, and the presence of transitional forms of meningopneumonitis virus from "intermediate forms" (intermediate bodies) to elementary bodies were described In spite of numerous intensive investigations by many workers, however, it may be safely said that formation of large bodies in an early stage has not yet been established in detail experimentally Speculation is used to fill in gaps It is worthy to note that the conspicuous presence of the invading virus particles contrasts with the disappearance of virus particles in infections of viruses of the pox group

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The Metabolism of Virus-Infected Animal Cells

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Introduction

Within the past few years studies of the virus host cell relationship have been undertaken by specialists in almost every area of biology. While, as biochemists, we look forward to the time when it will be possible to explain all of the observations in this field on a molecular basis, we are at present far from this objective. When more is known about nucleic acid and protein biosynthesis, for example, it should be possible to translate many of the observations of the immunologist and the cytochemist into biochemical terms. We have felt it necessary, therefore, to restrict this article in two ways. First, since considerations of immunology, epidemiology, and so on, are presented in practically every volume of reviews, none are included here. Without cataloguing a great number of such articles, it can simply be said that they range from discussions of a single virus disease, such as that on poliomyelitis by Melnick (80), to a review by Dulbecco (26), in which nearly every aspect of virus cell interaction except that of metabolism is considered. Second, we have also limited ourselves in more biochemical areas. Thus, studies of the

chemical nature of the viruses themselves have not been considered, for excellent reviews of this topic, the reader may turn to KNIGHT (59), PUTNAM (92) or SCHRAMM (108) Also, since we are concerned strictly with the effect of virus infection on the normal animal cell, we have omitted, for example, the insect viruses where little is known in this respect although there is a great deal of other information Again, since a considerable number of reviews are available dealing with the effects of alterations of host cell metabolism on virus growth, we have not included this material Also studies on the effect of inhibitors have been included only where a specific action on host cell metabolism has been demonstrated Such inhibitor studies are closely related to problems of viral chemotherapy and we refer the interested reader to reviews by ACKERMANN AND FRANCIS (4), HORSFALL AND TAMM (49), MATTHEWS AND SMITH (78), and PEARSON (89) Hormonal effects on virus infection also fall in this area, and such work has been reviewed by KASS AND FINLAND (57) and SCHWARTZMAN, ARONSON, TEODORU, ADLER AND JAHIEL (109) Nutritional effects on virus infection have been adequately dealt with by BAUER (11), and since he also reviewed the early work on the metabolism of virus infected animal cells, no attempt will be made here to give a historical development Finally, we have dealt only with those agents now generally accepted as viruses, omitting completely the psittacosis lymphogranuloma group *

Before embarking on the subject matter of this paper, comments on its organization are in order In the study of the animal virus host cell relationship, the problem is to deduce general concepts from limited experimental work on a variety of systems If this article dealt with bacterial rather than with vertebrate cells, it would involve, mainly, consideration of work with one species of cells, and one group of viruses, i e , *Escherichia coli* and the T phages The worker in the field of bacterial viruses can start with detailed concepts derived from these systems, and then attempt to elucidate the differences which characterize other virus host cell systems In the field of animal viruses this is not yet possible, and since we are unable to derive any general concept of metabolism in all virus infected animal cells, we are forced to present the details involved with a variety of viral agents

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* The nature of these microorganisms has been reviewed recently by WEISS (121) and WENNER (122)

Influenza Virus

1 Carbohydrate and Energy Metabolism

ACKERMANN (1) in 1951 examined some of the energy relationships involved in the propagation of the PR 8 strain of influenza virus in the chorioallantoic membrane * of two week old chick embryos Oxygen consumption of the infected membranes was equal to that of controls during an increase of about 10^3 in virus titer There was no increase in virus in the absence of oxygen or air In the presence of malonate, glucose uptake was not affected but there was a decrease in both respiration and viral growth The malonate was not virucidal, and was still effective when given 4 hours after infection However, malonate, although depressing viral synthesis, did not deprive the membrane of the ability to support viral growth, even after 24 hours incubation with the compound ACKERMANN also found that antimycin decreased respiration somewhat, and strongly inhibited viral reproduction, though glycolysis was little affected In other experiments, ACKERMANN AND JOHNSON (5) showed that dinitrophenol reversibly inhibited growth of influenza PR 8 in CAM, and that it was not virucidal When respiration was stimulated two fold with DNP (correlated with release of inorganic phosphate), almost no viral synthesis occurred It was concluded that the energy for virus synthesis in these systems was related to the mechanisms of oxidative phosphorylation of the host

EATON's observations (33), studying the growth of influenza strains PR 8 and Lee, and mumps virus on chorioallantoic and amniotic membranes of chick embryo, are in harmony with ACKERMANN's studies When the tissues were incubated in the absence of glucose before infection, virus multiplication was inhibited, but could be restored by the addition of glucose and, to a smaller extent, pyruvic acid and alanine Succinate, acetate, gluconate, glutamate and a number of Krebs cycle intermediates were inactive Diamidines, which increase aerobic glycolysis, were found to depress viral synthesis,

* Hereafter abbreviated to CAM

very strongly in the case of mumps virus With low concentrations, the effect was reversible When the diamidines were added 24 hours after infection, they were much less effective, since virus growth could then be prevented only by concentrations sufficient to stop cellular growth When dinitrophenol was added, respiration and glycolysis were increased but viral growth was depressed, both with deembryonated eggs and with membranes in culture EATON concluded that virus growth was dependent upon readily available energy sources and on transfer of energy by phosphorylation

DANIELS, EATON AND PERRY (23) in studying influenza infection in deembryonated eggs also found little growth of virus in the absence of glucose This is consistent with MILLS' observation that the deembryonated egg infected with Lee strain of influenza B takes up more glucose than the uninfected egg (81) Pyruvate and alanine were found to substitute for glucose in this system

LEVINE, BOND AND ROUSE (67) studied the substitution of pyruvate for glucose in influenza infected CAM Two strains of influenza A, WS which has no neurotropic effect in mice, and NWS which is uniformly fatal to mice were used Both viruses multiplied on addition of 0 1 per cent glucose to a basal salt test medium Substitution of 0 1 per cent pyruvate for glucose allowed only WS to multiply 0 02 per cent glucose plus 0 1 per cent pyruvate gave maximal hemagglutinin titers for both viruses It could be shown that while NWS infected the cells in pyruvate it was not appreciably synthesized Addition of any of a number of compounds various glycolytic intermediates, pyridine nucleotides, and adenosine mono, di, and tri phosphates increased the titer of NWS over that in pyruvate alone Thus pyruvate alone was unable to supply all the factors needed for synthesis of the neurotropic strain Nevertheless pyruvate plays an important part in the synthesis of influenza virus since WIELGOSZ (123) found that PR 8 infection of chick CAM causes an increase in the initial rate of pyruvate uptake between 30 and 50 per cent That the route of pyruvate metabolism may be significant is also indicated by ACKERMANN's study on the relation of the Krebs cycle to viral synthesis (2) He found that sub lethal doses of sodium fluoroacetate, which prevents oxidation of citrate, caused a marked inhibition of viral synthesis in the lungs of mice infected with PR 8 *in vivo*, and an accumulation of citrate in the same tissue Sodium fluoroacetate was not virucidal, and its effect was apparent whether it was given 15 minutes, 6 hours or 12 hours after inoculation In contrast, this

compound was found by MOGABGAB AND HORSFALL (83) to cause only a slight delay in the growth of PR 8 and Lee strains of influenza, and the Habel strain of mumps in chick embryo, and of PR 8 and the pneumonia virus of mice in mouse lung These workers concluded that the metabolic processes blocked by fluoro acetate are not essential for the multiplication of these viruses In the light of all the information available, it appears that the metabolic mechanisms of the host involved in the use of carbohydrate for the generation of phosphate bond energy are not only unaffected by viral growth but are most probably required for replication

2 *Phosphorus and Nucleic Acid Metabolism*

SELLERS (112) studied the uridylic and thymidylic acid content of lung after infection of mice with PR 8, using sufficient virus to cause death by the third day Synthesis of new virus was detectable within a few hours after infection but could not be correlated with the small changes found in the nucleotide content As the author points out, such data are difficult to interpret because of possible infiltration of the lung by other cells

Changes in phosphorus content and distribution have also been studied PARODI (88) examining the acid soluble phosphorus of chick embryo infected with influenza A, found a decrease in phosphocreatine of the infected tissue which was apparent in 4 hours and returned to normal after about 24 hours This may be related in some fashion to changed energy requirements in the early period of infection Inorganic phosphorus and acid soluble phosphorus also decreased, but neither as much, nor for as long as phosphocreatine The overall distribution of phosphorus in CAM of chick embryo infected with PR 8 was studied by COHN (16) Lipid phosphate decreased in the membrane and increased in the fluid In addition, there was more rapid uptake of P^{32} into the membrane, a small part of this being due to RNA The RNA DNA ratio was unchanged

LIU, BLANK, SPIZIZEN AND HENLE (74), and GRAHAM AND MCCLELLAND (44, 45) injected P^{32} into the allantoic cavity of the chick embryo and, on infection with PR 8, showed incorporation of P^{32} into lipid and nucleic acid fractions of the new virus HOYLE (51) also performed such experiments, and found that all of the isotope appeared in the soluble antigen fraction When the P^{32} containing

virus was reinoculated into CAM, it was found that after 1½ hours 20 to 40 per cent of the total P^{32} appeared in an isotonic saline extract of membrane in the form of low molecular weight phosphorus compounds and free nucleic acid, accompanied by small amounts of nucleoprotein and lipid The major part of the viral nucleoprotein P^{32} was in the membrane, and since a considerable amount of isotope appeared in molar saline extracts, it was presumably associated with nuclei HOYLE also used S^{35} methionine to prepare labeled virus and found that the isotope was incorporated into both the soluble antigen and the hemagglutinin fractions The results were somewhat the same as with phosphorus after 1½ hours about one-fourth could be recovered in an isotonic saline extract, and was presumably not intact virus S^{35} was extracted from the membrane very slowly by molar saline, and HOYLE found no evidence for specific association of S^{35} with the cell nuclei as such The results suggested only that a major part of the viral protein was associated with some insoluble cell component Observations such as these on the intracellular localization of virus parts should eventually be correlated with altered metabolic activities, although at present, the significance of such findings is obscure Further, one faces the general problem that the isotope distributions observed may well be those of labeled non infective particles in the virus population, as suggested by recent findings of TAYLOR AND GRAHAM (117) in studies of labeled poliovirus

3 *Amino Acid and Protein Metabolism*

Investigation of the amino acid and protein metabolism of influenza infected tissues shows no characteristic alterations KILBOURNE AND HORSFALL (58) found that infection of the chick embryo with influenza PR 8 and Lee, mumps, and Newcastle disease viruses, caused an increased protein concentration in the infected allantoic fluid (as measured by the turbidity developed on addition of 10 per cent trichloroacetic acid), which paralleled the increase in virus titer The protein involved was not viral and was immunologically the same in both normal and infected embryos As the authors point out, the phenomenon is not necessarily related to viral growth since the injection of broth or serum also brought about a marked increase in the protein content of the fluid KALTER (55) examined the amino acids present in the allantoic fluid proteins after infection with PR 8 or

Lee strain of influenza virus Small molecules were dialyzed out of the allantoic fluid and the non dialyzable fraction was then hydrolyzed and analyzed for amino acids Increased concentrations of aspartic and glutamic acids, isoleucine, leucine, methionine, phenylalanine, threonine, serine, tyrosine, valine and tryptophane were found with both viral infections

JOHNSON, KEMPF AND BERGEIM (53, 54) examined the pattern of free amino acids in chick CAM after infection with PR 8, and found seemingly significant decreases in the lysine and histidine content These were not detected in similar experiments with mumps Since the mumps virus causes only minimal tissue damage it was concluded that the decreases were due to inflammation rather than to infection SHULLS AND RIGHTS (113) in similar experiments, examined both the peptide and amino acid composition of the free amino acid pool of PR 8-infected CAM Their data suggested that glutamic acid is abnormally increased in tissues synthesizing large amounts of virus and that as viral replication decreases, the concentration of glutamic acid returns to normal MILLS (81), using the deembryonated egg infected with Lee strain of influenza B, examined the uptake of various amino acids and concluded that the infected cell has an increased requirement for cysteine, histidine, and methionine

RAFELSON AND ARNOFF (93) investigated the conversion of C^{14} glucose to various amino acids by CAM infected with PR 8 After 4 hours incubation glutamic acid aspartic acid serine, alanine, and glycine were found to contain C^{14} At 8 hours, more C^{14} had been incorporated into the infected than into the control system, and this increase persisted at a linear rate for 8 more hours At 24 hours the rate in the infected system declined while the control continued at a linear level In spite of the increased incorporation of C^{14} into the amino acids upon infection, there were no significant changes in the total amount of amino acids in the two systems Consequently, the rates for both formation and use of amino acids increased upon infection The increase in viral titer and C^{14} incorporation were parallel, but the authors feel they are not necessarily related As would be anticipated on the basis of the findings described above, virus infected tissues which had been pre labeled with glycine and alanine showed a greater loss of activity from the free amino acid fraction than did controls The loss of activity from the tissue protein component was unchanged by infection, as was the rate of incorporation of isotopic lysine, histidine, alanine, and glycine into the membrane proteins

4 *Other Studies*

One study using inhibitors is of interest since it suggests a correlation between the various stages of viral synthesis and the metabolic activities of the host cell ACKERMANN AND MAASSAB (7) infected CAM with influenza strain PR 8 and inhibited viral growth with either AMPS [α amino α (p methoxyphenyl)methane sulfonic acid] (a tyrosine analog), or methoxinine (a methionine antimetabolite) The actions of these inhibitors on virus synthesis were found to be sequential the first phase, adsorption and penetration, is inhibited by AMPS, but not by methoxinine The second phase proceeds in the presence of AMPS but is inhibited by methoxinine No mature virus is produced during this time The third phase, production of mature virus, is insensitive to both inhibitors And finally, the release of virus is sensitive to inhibition by AMPS

An example of one other type of study, although not directly connected with our topic, should be mentioned at this point COLVILLE, DUNBAR AND MORGAN (20) examined the ability of a variety of chick embryo tissues to support influenza growth in culture media Very young tissues were found to be incapable of supporting viral growth, whereas the susceptibility changed markedly in two weeks At this age (2 weeks), some embryonic tissue (heart, skin, muscle, and liver) supported a small degree of viral growth while other tissues like lung allowed excellent growth Such information will be very valuable when correlations with the metabolic changes undergone by differentiating tissue become possible At present we know little about the factors involved in susceptibility to viral infection, and extension of studies of this kind may offer one approach to these problems

5 *Summary*

The influenza viruses have been studied mainly in the intact animal or with tissues *in vitro* In many instances it is difficult to be certain of the site of the changes observed and to know to what tissues or cells they should be ascribed Consequently we have little precise information on the relationship of influenza virus infection to host cell metabolism Normal operation of carbohydrate metabolism and the associated phosphorylative mechanisms seems necessary, as

well as the existence of the normal cellular mechanisms for nucleic acid and protein synthesis Such changes as do occur during virus growth, at the present time, can be described only in terms of alterations of the pattern of incorporation of inorganic P^{32} The lipid metabolism of cells infected with influenza virus has not been examined

Encephalitis Viruses

1 Carbohydrate and Energy Metabolism

HUANG (52) in 1943 examined St Louis encephalitis, Jungeblut Sanders mouse virus, and Western equine encephalitis for their effects on various tissues in culture Mouse embryo brain was used for the first two viruses and chick embryo skeletal tissue for the last He found that virus production in these tissues could be correlated with decreased production of acid, using phenol red as an internal pH indicator This has been the basis of a variety of viral detection techniques developed since then, and BROWN (14) has modified it for the measurement of Western equine encephalitis and its antibody

PEARSON AND WINZLER (90) studied the relationship of carbohydrate metabolism to the growth of Theiler's GD VII virus in minced day-old mouse brain Maximal growth of virus had no effect on the oxidative or glycolytic metabolism of the tissue, and virus was still able to grow even when the tissue had been incubated prior to infection until the oxygen consumption, glucose utilization, and lactic acid production were very low The authors concluded that the rate of carbohydrate metabolism was not of prime importance in this system

WATANABE, HIGGINBOTHAM AND GEBHARDT (119) found that sodium fluoroacetate prolonged the survival time of mice infected with Eastern equine encephalitis virus Although the compound had no effect on virus infectivity nor on the susceptibility of the animal to the virus, the authors associated its action with inhibition of citrate oxidation in accord with ACKERMANN's findings in the case of influenza infections (2)

RAFELSON, WINZLER AND PEARSON (96) examined the effect of Theiler's GD VII encephalomyelitis on the incorporation of labeled glucose carbon into minced day old mouse brain and found that viral growth increased the incorporation of labeled carbon into protein and decreased the incorporation into lipid

2 *Phosphorus and Nucleic Acid Metabolism*

In studies with minced day-old mouse brain infected with Theiler's GD VII virus RAFELSON ET AL (96) found that in the presence of P^{32} the specific activity of all fractions of both infected and uninfected mouse brain increased, reaching a maximum between 24 and 48 hours, and then decreased The relative specific activities of all the phosphorus fractions of infected cells, except the DNA fraction, were increased over the controls The DNA was not significantly different from the controls The authors suggest that these findings may be taken as an indication that the virus is of the RNA type

A somewhat similar experiment using P^{32} was carried out by LEVY AND SNELLBAKER (72) with an encephalomyocarditis virus infecting Ehrlich ascites tumor cells growing in mice No difference was found between the RNA and DNA composition of the infected cells and those of the controls When the loss of radioactivity from the cells was followed, it was found that the specific activity of all the phosphorus fractions of infected cells decreased more rapidly than those of uninfected cells

RAFELSON, PEARSON AND WINZLER (94) examined the P^{32} uptake and oxygen consumption of minced mouse brain during the growth of GD VII virus They found that the virus grows only in minced mouse brain of one-day old animals, with maximal production 12 to 24 hours after infection They also found that oxygen consumption diminishes rapidly in tissues from animals older than 1 day, and hence suggested that virus growth requires the sustained aerobic metabolism characteristic of 1-day old tissue P^{32} incorporation after infection was found to follow a similar pattern, decreasing as the animal became older until there was no difference in uptake between infected and control systems

RAFELSON, PEARSON AND WINZLER (95) found that 5-chlorouridine inhibited both viral synthesis and P^{32} uptake into the RNA of GD VII infected minced brain tissue, whereas it had no effect on tissue from controls The authors suggested that RNA synthesis associated with viral multiplication is more sensitive to inhibition than is the synthesis of normal RNA The increased incorporation of isotope into the lipid phosphorus fraction following infection still appeared in the presence of 5 chlorouridine

When the P^{32} distribution was examined with respect to particle size by MOLDAVE (84), it was found that the increased uptake was

most pronounced in the protein and nucleic acid of the smallest particulate fraction of the cell

3 *Amino Acid and Protein Metabolism*

The amino acid metabolism of the GD VII infected mouse brain has been examined by RAFELSON, WINZLER, PEARSON AND MOLDAVE in a series of papers (85, 97, 124) Infected minced day-old mouse brain had a lower lysine and histidine concentration, and a higher cystine concentration than uninfected tissue The C^{14} of uniformly labeled glucose was incorporated into all the amino acids of controls except proline and methionine on 24 hour incubation The incorporation into most of the amino acids was increased by infection, but that into lysine and histidine was decreased These two amino acids, which are found in less than normal concentrations in infected day-old mouse brain, were, moreover, shown to be inhibitors of viral growth and P^{32} uptake in this system These authors found that in mouse brain *in vitro*, intermediates in glucose metabolism can be used to synthesize amino acids (all the essential amino acids, for example) not normally produced by the intact animal When C^{14} labeled acetate or formate was used in place of glucose no isotope was found in lysine, and infection with the GD VII virus depressed incorporation into histidine

4 *Summary*

Infection of cells by the encephalitis viruses apparently leaves the normal energy-yielding cellular processes intact There are indications of a redirection of carbon metabolism following infection, inasmuch as the C^{14} incorporation into cellular lipid decreases while that into protein increases A redirection of phosphorus metabolism is also suggested by alterations in incorporation of P^{32} into various chemically and physically distinct cellular fractions The particular type of nucleic acid in the virus may explain the circumstance that there are changes in the activity of cellular RNA but not DNA There are no marked alterations in the use of carbohydrate carbon for the biosynthesis of amino acids except in the case of lysine and histidine The metabolism of these two amino acids appears to be closely associated with viral multiplication

Poliomyelitis Virus

1 *The Metabolism of Cells in Tissue Culture*

The viruses considered up to now have been, to a great extent, studied in complex hosts—the mouse, chick embryo—or in minced brain tissue used with a complex maintenance medium immediately after excision These are frequently the methods of choice for investigation of virus host interactions However, in the past few years the simplification of techniques for growing cells in culture has given a great impetus to the study of effects of virus on metabolism at the cellular level Enzymatic changes and their causes, the intracellular sites of viral synthesis, sources of energy, and biosynthetic pathways are studied with more simplicity in an isolated system where we have reasonable assurance of the cell types involved and of the degree of infection produced Needless to say, all the work done with cells in tissue culture has not been of this character, but the solution of technical problems, such as quantitative virus assay, suggests that objectives such as these may be approached It seems reasonable to assume that work of this kind will help in understanding the more complex situations found in the virus infected animal, and we have felt it advantageous to include data on the metabolism of cells in tissue culture in the discussion which follows

In view of the growing number of applications of tissue culture technique, we refer those interested in possible uses to the comprehensive review by ROSS AND SYVERTON (104)

2 *Carbohydrate and Energy Metabolism*

ROBBINS, ENDERS AND WELLER (102) in 1950 described the cytopathological changes and decreased acid production following infection of human embryonic skin muscle tissue with Lansing or Brunhilde strain poliovirus These changes have been observed after infection with many strains of poliomyelitis virus, and are the basis of a number of methods for virus and antibody assay (73, 103, 106) The normal acid production of cells has been used by RAPPAPORT (100) to estimate the number of cells in a monolayer culture by determining the color change in phenol red solution during a standard period of

time In this study it was shown that the hydrogen ion production of cells under the conditions of measurement remained constant for short periods of time, although the endogenous carbohydrate content varied from 11 to 70 micrograms per million cells It was suggested that the excretion of hydrogen ions with some anion is a primary function of the cell and is maintained as long as the cell retains its integrity, even at a minimum metabolic rate

FRANKLIN, DUNCAN WOOD AND RHODES (37) found that the utilization of glucose by human embryo brain and cord infected with the Lansing virus was less than normal, glucose utilization had usually declined considerably below half the normal value after 3 days

ZWARTOW AND WESTWOOD (125), investigating the factors affecting the growth of a variety of mammalian tissues in culture, found that maximum growth (and minimum glycolysis) occurred with oxygen concentrations greater than 10 per cent This is of interest in connection with the observation of GIFFORD, ROBERTSON AND SYVERTON (38) that in *HeLa cells infected with* Type 1 Mahoney strain poliovirus, maximal yields of virus were obtained between pH 7 2 and 7 8, the range of maximum oxygen uptake

Results of this kind suggested that oxidative metabolism was essential for the production of poliovirus in tissue culture However, GIFFORD AND SYVERTON (39 40) studied the energetic requirements for virus multiplication and found that both HeLa cells and monkey kidney permitted replication of poliovirus under strict anaerobic conditions While the latent period before viral release was prolonged under anaerobic conditions the yield per cell was the same as in the presence of oxygen or air Infection of HeLa cells by Type 1 Mahoney *strain caused no change in* the oxygen consumption of aerobic cells or the acid production of anaerobic cells until cytopathology was evident The authors suggest that HeLa cells are apparently facultative aerobes with regard to the means of energy production for virus synthesis In contrast to the results of GIFFORD AND SYVERTON, LEVY AND BARON (69) found that within one half to one hour after infection of monkey kidney by Saukett strain of type 3 virus, lactic acid production was increased and remained above the control rate for 6 to

within an hour after infection—long before appearance of new virus or cytopathology It is possible that the differences in the results of

FISCHER (82) showed that infection of HeLa cells by Mahoney virus caused an increased P^{32} uptake into the nucleic acid and lipid fractions, apparent 1 hour after infection Virus titer and nucleic acid labeling increased simultaneously

The total RNA phosphorus of HeLa cells is approximately 30×10^{-10} mg per cell, and the DNA phosphorus about 20×10^{-9} mg per cell (41,76) MAASSAB, LOH AND ACKERMANN (76) found that the nuclear RNA was 1½ times the cytoplasmic RNA Infection of HeLa cells with Mahoney strain poliovirus caused approximately 250 per cent increase in the amount of cytoplasmic RNA in 7 hours relative to the uninfected cell The nuclear RNA and DNA showed little change All these fractions from infected cells showed an increased P^{32} uptake to have taken place (approximately 175 per cent that of the uninfected cells) during the first hour after infection The incorporation into DNA decreased with time, while that into cytoplasmic RNA increased The nuclear RNA showed the highest rate of incorporation 2 hours after infection, the specific activity of this fraction was two to three times that of the cytoplasmic RNA Since the nuclear RNA and DNA remained constant in amount, the increase in P^{32} suggests an active nuclear turnover rather than net synthesis of nucleic acid This, the authors believe, is associated with the protein synthesis following virus infection The changes in RNA labeling occurred before virus was demonstrable, and were maximal before the virus reached maximal titer

In a later report, LOH, PAYNE AND ACKERMANN (75) found that the cytoplasm of infected HeLa cells contained increased quantities of polymerized nucleotides The usual four bases of RNA were present in larger amounts but in the same proportions as in normal cytoplasm Examination of 3 cytoplasmic fractions with respect to RNA, protein, and virus content indicated that the bulk of the RNA and virus was found in association with the smaller cytoplasmic particulates

GOLDFINE ET AL. (42) studied the incorporation of uniformly labeled C^{14} cytidine into HeLa cell RNA and DNA during infection In the period from 1 to 11 hours after infection with Saukett strain of type 3 poliovirus, incorporation of cytidine into RNA was 60 to 80 per cent of the control level During the second half of the experimental period (from 5½ to 11 hours) incorporation of cytidine into RNA was approximately the same in the infected and control systems, while the incorporation into the DNA of infected cells was only 10–25 per cent that of the controls This indicates a depression of

either DNA synthesis or turnover while RNA, being used for viral synthesis, continues to be metabolized by the cell Whether this incorporation represents actual RNA synthesis or simply turnover was not determined, but from the work of ACKERMANN's group, it seems safe to conclude that net synthesis of RNA was involved

A brief summary of recent work concerned with the metabolic stability of the nucleic acids in normal cells adds to the picture presented thus far GRAHAM AND SIMINOVITCH (46) prepared P^{32} labeled strain L cells and kept these growing logarithmically in non radio active medium by the periodic addition of new medium They found an initial small loss of P^{32} from the RNA fraction (presumably due to handling and transfer) but no loss of P^{32} from DNA Calculation of the concentrations of P^{32} in the cells showed conservation of RNA phosphorus for 6 generations and of DNA phosphorus for 9 generations There was no renewal of DNA phosphorus or the bulk of RNA phosphorus during logarithmic growth THOMPSON, PAUL AND DAVIDSON (118) studied the stability of nucleic acids in strain L cells growing slowly near the maximum population level Cellular nucleic acids were labeled with C^{14} from formate and the loss of activity from DNA and from nuclear and cytoplasmic fractions of the RNA was followed Both types of RNA showed some turnover Very little isotope was lost from the DNA and it was suggested that although turnover of DNA occurs, it is obscured by the reincorporation of its breakdown products

Another approach to the problem of altered nucleic acid metabolism upon infection with poliovirus is typified by the work of KOVACS (61–65) who examined several enzymic activities which are directly and indirectly connected with nucleic acid metabolism Monkey kidney epithelial cells were infected with Mahoney, Saukett, or MEF 1 viruses, and the cultures examined on the sixth day of infection for changes in enzymatic activity Acid and alkaline phosphatase, 5-nucleotidase, acid RNAase, and acid and alkaline DNAase decreased in activity in the infected system, while alkaline RNAase increased in the infected system The depression of DNAase activities appears to be due to an inhibitor produced or released after infection, since virus infected culture fluids inhibit the activity of other DNAase
It was found that the acid phosphatase activity of infect ce
cally disappeared after an initial increase It is felt
increase in acid phosphatase during maximal
cates a role for this enzyme in early synthetic

KOVACS also suggests that polio infection is essentially a disturbance of RNA metabolism with the cellular DNA remaining constant, at least during early phases of the infection In terms of the operation of normal biosynthetic mechanisms however, we can look on polio infection equally well as a disruption of DNA metabolism with the RNA component remaining intact

4 *Amino Acid and Protein Metabolism*

EAGLE AND HABEL (29) observed (as cited earlier) that glucose, glutamine, and salts are the only requirements for poliovirus synthesis in the HeLa cell DARNELL AND EAGLE (24, 25) found that vitamin B_6 depleted cells were unable to produce virus from glucose, glutamine, and salts Loss of this capacity was associated with changes in the free amino acid pool of the cells When cells were starved, glutamine was found to disappear within 6 hours from the free amino acid pool While the role of glutamine is uncertain, EAGLE, OYAMA, LEVY, HORTON AND FLEISCHMAN (31) suggest that it provides an essential transaminating mechanism on the basis of the sparing action of added non essential amino acids

Study of other human cell lines demonstrated that glutamine was required for growth, although it could be replaced by high concentrations of glutamic acid (30) Other amino acids and a variety of purines, pyrimidines, and ammonium salts decreased but did not abolish the glutamine requirement Monkey kidney cells need the same amino acids as HeLa cells, but their glutamine requirement can be satisfied by glutamic acid, aspartic acid, or asparagine (28)

RAPPAPORT (99) studied the capacity of monkey kidney cells to support poliovirus synthesis in a synthetic medium containing glucose, cysteine, isoleucine, histidine, arginine, lysine, methionine, threonine and salts Cysteine was required for successful growth, and could replace the entire amino acid supplement on infection of kidney cells with Type 1 Brunhilde strain poliovirus The salts, moreover, included no ammonium compounds Consequently, viral multiplication in these cells, just as in HeLa cells, apparently uses only some of the biosynthetic systems normally required for growth

In studying the growth of poliovirus in HeLa cells, ACKERMANN, RABSON AND KURTZ (8) found that there was no parallel between cellular injury and viral multiplication A latent period of 4 to 5 hours

was followed by virus release over 6 to 7 hours Changes in cellular staining could be detected before cytopathology was far advanced and before the release of the major amount of virus Virus production and cellular multiplication were markedly inhibited in the presence of fluorophenylalanine, and these effects could be completely reversed by phenylalanine Fluorophenylalanine, however, did not retard the occurrence of cytopathological changes

LEVY AND BARON (69, 70) examined the uptake of glycine 2 C^{14} by normal and Saukett infected monkey kidney cells 30 minutes before harvesting of the cells Infection inhibited the uptake of the isotope into the lipid fraction of the cells most strongly Uptake into the nucleic acids was least affected The changes were apparent within 1 hour after infection, and long before the appearance of new virus

In a series of studies pertinent to those described above, EAGLE AND COWORKERS (32, 68) have demonstrated the roles of the various amino acids required in the metabolism of cultured cells Glutamine, uniformly labeled with C^{14} and having N^{15} in the amide group, was incorporated directly into cellular protein without degradation the whole of the protein glutamine being derived from the glutamine of the medium Glutamine and glutamic acid acted independently in protein biosynthesis, neither being a precursor of the other Neither amide nitrogen nor ammonia were α amino nitrogen precursors The carbon chain of glutamine was the precursor of aspartic acid and proline The C^{14} of uniformly labeled phenylalanine and tyrosine was incorporated almost entirely into protein as the corresponding amino acid The non essential amino acids, when not supplied in the medium, were found to be highly concentrated in the cells, as they probably are during viral synthesis in a glucose glutamine salts medium

5 Other Studies

In the past several years a number of studies have been directed toward localizing the intracellular site of poliovirus synthesis SCHWERDT AND PARDEE (110) found that about 4/5 of the virus in the nervous system of Lansing infected cotton rats was free in the sub microscopic particle fraction the microsomes KAPLAN AND MELNICK (56) obtained results similar to those of SCHWERDT AND PARDEE since cells from the central nervous system of mice paralyzed by polio infection had a higher concentration of virus in the nuclear fraction

uptake was increased into all cellular fractions, least into the lipids. Glycine C^{14} incorporation was increased *only* into the acid soluble fraction. These changes depended on the presence of infective virus. Monkey kidney, which exhibits no early pathology, showed the same changes.

PEREIRA (91) and EVERETT AND GINSBERG (34) have recently been able to separate a protein factor from adenovirus particles which will cause reversible clumping and rounding of HeLa cells without nuclear alterations, and which does not affect viability or growth. HeLa cells undergo these cytopathological changes soon after infection with adenovirus.

3 *Summary*

In the absence of other evidence, we can assume that the adenoviruses use the carbohydrate metabolism of the host cells as the energy source for virus synthesis. In contrast to a number of other viruses, these cause increased acid production by the infected cells. Infection stimulates the turnover of RNA and DNA phosphorus but apparently not RNA and DNA synthesis. P^{32} uptake into all cell fractions is increased by infection, while glycine incorporation is increased only into the acid soluble fraction of the cell.

Other Viruses

A wide variety of other viruses have been studied for their effects on host metabolism, but such investigations have not been nearly so extensive as those presented above, and the data are best considered in terms of the effects on specific areas of metabolism.

1 *Carbohydrate and Energy Metabolism*

SMITH AND KUN (115) examined the effect of myxoma, fibroma, Rous sarcoma, herpes simplex, vaccinia, Newcastle disease, and swine influenza on chick embryo CAM. Infection with any one of these left the oxygen uptake of the membrane essentially unchanged, but increased glycolysis.

NELSON AND DE BURGH (86) determined changes in enzymic activities in ectromelia infected and regenerating mouse liver. They examined the ratio of uricase to acid phosphatase in various fractions, and found it to be approximately the same in normal and regenerating tissue. Virus infection caused a lowered ratio in the "heavy mitochondrial", and supernatant fractions. Such a finding is difficult to evaluate, since it may be either a primary response to infection, or simply a result of virus interference with more fundamental processes.

2. *Phosphorus, Nucleic Acid and Protein Metabolism*

BLANK, KAMIDA AND LIU (13) studied the P^{32} partition in de-embryonated eggs infected with herpes simplex and vaccinia. Autoradiograms of the infected embryos showed that the P^{32} content increased in blood vessels, CAM pocks of vaccinia, and points of mechanical injury. After infection with either virus the concentration of P^{32} in the amniotic fluid increased 10 to 20 fold in 48 hours. COOPER (21) studied the path of phosphate transfer in monolayers of minced chick embryo tissue infected with vesicular stomatitis virus. He found that phosphorus entered the cell via an inorganic pool (apparently in equilibrium with external phosphate) and was transferred to larger molecules or to the organic acid soluble phosphate fraction. When virus infection was synchronous (i.e., one step growth) there was no detectable change in the rate of gain of P^{32} by the acid soluble phosphate, lipid phosphate or RNA, until uptake ceased at about the middle of the time of exponential release of virus. In a continuation of this study on monolayers of minced chick embryo cells, COOPER (22) concluded that an osmotic barrier near the cell surface regulated the reciprocal exchange of inorganic phosphate between the medium and some component of the acid soluble phosphorus fraction. The adsorption, penetration, and release of vesicular stomatitis virus appeared to occur without any damage to the mechanisms involved in this phosphate exchange.

Nucleic acid and protein metabolism have been examined in a variety of virus host systems. RANDALL AND MOORE (98) studying the infection of HeLa cells with equine abortion virus, found that after 48 hours incubation, infected cells had a significantly increased protein and DNA content, while the RNA content remained normal. NEWTON AND STOKER (87) infected HeLa cells with herpes virus,

which has only 6 per cent the infectivity toward this cell as it does toward CAM Total nucleic acids, DNA and RNA were determined Nine hours after infection the DNA of infected tissue was 40 per cent above the control value After a stationary period of 10 to 12 hours there was further increase of DNA The RNA of both control and infected cells was essentially constant After 3 to 4 days those cells which showed pathological changes had about twice as much DNA as the controls and contained practically no RNA

ACKERMANN AND FRANCIS (3) studied embryonated eggs whose CAM had been infected with herpes simplex Such infection is followed by growth of virus in the CAM, and the liver, heart, spleen and brain of the embryo Three days after infection, the livers and hearts of infected embryos were 30 to 40 per cent larger than those of normal embryos The number of nuclei, the protein nitrogen, total nitrogen and water content per gram of these tissues were the same in normal and infected embryos, leading to the conclusion that the number of cells in the infected organs had increased even though the total weight of the infected embryo was less than that of the normal embryo Succinoxidase activity of both normal and infected livers was about the same Infected heart showed, however, about 27 per cent less succinoxidase activity (on a wet weight basis) than normal heart tissue The increased size of organs of the infected embryo caused the amount of α ketoglutaric oxidase of heart and liver, and the succinoxidase of heart to be approximately equal in both the normal and infected systems In contrast to heart, the liver succinoxidase increased by about 50 per cent The total nucleic acid content of heart and liver in the infected system increased 24 per cent above the control value (on a dry weight basis) with no change in the DNA/RNA ratio ACKERMANN AND KURTZ (6) carried this study further by fractionating the cellular components of the livers of infected embryos 80 per cent of the new virus was found apparently free in the cytoplasm while 16 per cent was tightly bound to mitochondria Mitochondrial nitrogen per gram of liver decreased 30 per cent after infection, due to degeneration of liver cells The physical relationship of virus and mitochondria observed in this study, together with the biochemical relationship observed in the previous study (3) (changes in enzymatic activities associated with mitochondria were observed after infection with herpes virus), led these authors to suggest that the mitochondria are a site of herpes virus synthesis

REISSIG, BLACK AND MELNICK (101) infected a human cell line,

Hep-2, with the Edmonston strain of measles in two different media In Ender's medium (deficient in glutamine), infection caused formation of a few huge syncytia, with some of the cells containing several hundred nuclei In Eagle's medium (containing glutamine), spindle-shaped cells appeared in a net rather than in a sheet after infection The addition of glutamine to Ender's medium resulted in a marked reduction in giant cell production and in the transformation of most of the cells into forms resembling those obtained in Eagle's medium Glutamine is not essential for virus multiplication in this case, but the mechanism whereby its presence or absence is reflected in different patterns of cytopathology after infection is a subject for future study

3 *Summary*

Very little can be said of fragmentary results such as those just presented They suggest that energy metabolism is left reasonably intact after infection, and that the aberrations in cellular nucleic acid metabolism are a reflection of the type of nucleic acid in the infecting particle Most of the data cannot be related to the normal metabolic activities of the host simply because too little is known about the latter

Conclusion

GOTTSCHALK (45) has recently suggested that viral nucleic acid (or some part of it) acting as a genetic determinant, is built into the host nucleic acid and protein synthesizing enzyme systems as a coenzyme In this way the viral sequences of component parts are impressed on the nucleic acid and protein produced by the cell If we examine the great variety of observations recorded here with this suggestion *in mind*, two things become apparent First, none of the data is in conflict with the idea—some tend to give it support Secondly, specific effects of virus infection on host cell enzymes should eventually be demonstrated in nucleic acid and protein biosynthesis

The evidence that the nucleic acid of a virus is the infectious agent has been reviewed in detail by COLTER (17) Suffice to say here that infectious preparations of ribonucleic acid have been isolated from polio infected tissue by COLTER ET AL (19) and ALEXANDER ET AL (9), from West Nile encephalitis infections by COLTER ET AL (19),

from Mengo encephalitis infections by COLTER ET AL (18), and from Eastern equine encephalomyelitis by WECKER AND SCHÄFER (120) These findings are of interest in relation to the entire problem of nucleic acid synthesis, and seem particularly relevant since primer nucleic acid is required in those nucleic acid synthesizing enzyme systems known at present (47, 60)

From the studies reviewed here, it appears that the energy pro ducing systems of the infected cell must be relatively intact This is also true for the protein and nucleic acid synthesizing systems of the infected cell Numerous observations indicate that various corollary activities (carried on by the cell during normal growth) can be dis pensed with during virus synthesis

When we turn to the second point mentioned above, our inability to show specific effects of a virus on host cell enzymes is undoubtedly due to the complexity of the systems used Whether the intact animal or a cell culture is the infected host, the results are nearly always the *means* of unknowns—the *mean* of different kinds of infected cells, the *mean* of infected plus uninfected cells, etc The ideal system is one in which cells, uniform in respect to type and physiological state, can be synchronously infected by a completely infective virus preparation Among the recent advances toward this goal we may cite the work of MARCUS, CIECIURA AND PUCK (77) on clonal growth of cells, and that of MCLIMANS' group on growth of cells in suspension (79) With pure cell lines, easily grown in quantity, fundamental metabolic studies such as that of SALZMAN, EAGLE AND SEBRING (107) on the biosynthesis of nucleic acid bases in HeLa cells, should be capable of considerable amplification

Other advances which hold future promise are concerned with the preparation of homogeneous virus samples With the development of highly efficient assay systems such as that described by FOGH AND LUND (36) for poliovirus, future work should be much more quantita tive in nature, and newer techniques for the purification of viruses such as those described by HOYER AND COWORKERS (50) will give purer preparations in quantity without requiring excessive time and material

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strains were actually considered by other investigators to be a special type of Coxsackie group A virus (LENNARZT ET AL., 1957, TYRRELL ET AL., 1956, WOHLRAB ET AL., 1957, DALLDORF, 1958) Among types 1, 2, and 3 polioviruses some strains exist which are of low virulence for monkeys (SABIN, HENNESSEN AND WINSSER, 1954, KOPROWSKI ET AL., 1954, MELNICK, 1954) At the same time existence of especially virulent (highly neuroinvasive) epidemic strains of polioviruses in one and the same immunological type was repeatedly suggested (CHUMAKOV ET AL., 1951, 1955) Type 2 poliomyelitis virus was found capable under certain conditions of producing myositis in rodents (ARONSON ET AL., 1953, 1956) There are many examples of other variations of the pathogenic properties of different viruses

Therefore the time apparently has not yet come for a final classification of enteroviruses and especially of the many facultative and obligate neurotropic viruses isolated in connection with investigations of poliomyelitis and diseases with similar clinical picture

Under these conditions any discussion on the nature of the Karaganda strains must consider all their properties, including the capacity of these viruses to produce paralytic poliomyelitis in monkeys, cotton rats and probably human beings (though for the latter it would be desirable to have additional data and confirmations in more extensive studies and in a number of countries)

In the material presented below, it is shown that the ability of AB IV viruses to cause paralytic poliomyelitis is not accidental, transitory or gradually decreasing as the viruses are passed in susceptible animals Neurotropism of these strains for monkeys and cotton rats is no less stable a property than myotropism for suckling white mice On the contrary it was shown that tissue culture adapted AB-IV virus after 27 passages in kidney cultures lost its ability to infect suckling white mice, but preserved its pathogenicity for monkeys

Neurotropic properties were also revealed in investigations of the prototype strain WP of Coxsackie A7 virus (VOROSHILOVA ET AL., 1957) And though the question has not yet been settled on the identity of AB-IV and A7 viruses, we believe there are good reasons to exclude A7 virus from the Coxsackie viruses because of its neuro tropism for monkeys and cotton rats A suggestion was made to place poliomyelitis like AB IV and A7 viruses into an intermediate position between the true poliomyelitis viruses and the Coxsackie group of viruses (VOROSHILOVA, 1957, 1958, HORSTMANN AND MANUELIDIS, 1957, 1958)

monkeys and adult rodents cannot be considered adequate enough for some viruses of the group (DALLDORF, MELNICK AND CURNEN, 1959) For example, in addition to Coxsackie A7 virus, *under certain* conditions Coxsackie A14 virus produced in cynomolgus monkeys poliomyelitis like morphological changes in the spinal cord, although there were no clinical signs of paralysis, and Coxsackie A1 virus caused flaccid paralysis *in adult white mice* (DALLDORF, *1957) It is possible* that, as passages of Coxsackie viruses will be done more extensively and their pathogenic spectrum studied in more detail, additional data will be obtained on acquired or existing neurotropism of these viruses Even under these conditions AB IV virus is still *very different* from other Coxsackie viruses including A14 and A1, because of its unusually high and regular paralytogenic activity for monkeys and cotton rats

We are as yet not quite sure that A7 virus strains are completely identical with the Karaganda AB IV and other strains because, despite the great similarity between them, some differences may be noted, mainly in the intensity of neurotropism for monkeys and cotton rats Such a point of view is in keeping with the existence of quite independent infections, such as Russian spring summer ence phalitis, louping ill, Omsk hemorrhagic fever, Kyasanur forest disease and others, in which the causative viruses isolated have very many similar immunologic and pathogenic properties and yet fundamental differences exist in the clinical picture and epidemiology of the diseases Therefore we may expect other cases of close immunologic similarity between different kinds of clinically independent viruses Further comparison of serological, epidemiological, clinical and experimental data between the Coxsackie A7 viruses and Karaganda strains are obviously required

That revision of virus classification is quite natural is evidenced by the extension of knowledge on pathogenic and other properties of viruses Among viruses of the ECHO group some members were recently revealed as having properties which bring them close to either the Coxsackie group A viruses or to poliomyelitis viruses For example, some ECHO 9 strains isolated during an epidemic of aseptic meningitis in the European countries in 1956–1957 were found to be similar to Coxsackie group A viruses in their pathology for suckling white mice (MCLEAN AND MELNICK, 1957, HENNESSEN, 1957, BAUMANN ET AL, 1957, GARNETT ET AL, 1957, JUST AND BERGER, 1958, KRECH, 1956, SAUTOV, 1957) However these same

Until 1956 our laboratory for certain reasons did not work with prototype and other Coxsackie viruses, and we were not able to study antigenic relationships between Karaganda strains and the Coxsackie group of viruses It should be mentioned that the circumstances of isolation of Karaganda strains and their pathogenicity were such that the possibility of their relationship to the mild Coxsackie group was not suspected

In 1957 the first report by JOHNSSON AND LUNDMARK (Sweden) appeared indicating a cross immunologic relationship between AB IV and Coxsackie A7 viruses by the use of mouse neutralization tests This was confirmed in the U S A by HABEL AND LOOMIS (1957), and later by HORSTMANN AND MANUELIDIS (1957, 1958), as well as in our laboratory when in 1957 it became possible to work with prototype Coxsackie viruses At the same time the above workers found that the AB IV virus with great regularity produced myositis in suckling white mice, and also in suckling Syrian hamsters (IONESCU MIHAIESTI ET AL, 1957) The last authors also observed a higher susceptibility of cynomolgus monkeys to AB IV virus as compared to rhesus monkeys, and found lesions characteristic for poliomyelitis in the spinal cord neurons of monkeys infected with AB IV virus

In 1957–1958 in our laboratory evidence was obtained that the prototype WP strain of Coxsackie A7 virus was pathogenic for monkeys At the same time HABEL AND LOOMIS (1957) made the very important observation that monkey neurotropic A7 virus could be obtained from human stools which had earlier yielded strains pathogenic for suckling white mice and possessing marked myotropism

HORSTMANN AND MANUELIDIS (1958) made serial passages of AB IV virus in monkeys and obtained valuable histopathological and serological data characterizing the experimental infection more completely

Experiments of Russian as well as American and Rumanian investigators showed that in a number of monkeys infected with the AB strain, the histologic lesions were indistinguishable from those of poliomyelitis while in other cases there were lesions in areas not typical for poliomyelitis

Of practical importance is the fact that in DR K HABEL'S laboratory (U S A) the AB IV virus was adapted to monkey kidney cells, which made possible broad serologic investigation of the infection with this variant of the virus

After the work of the Russian investigators who demonstrated

History

The following is the short history of investigations of group A7 AB IV viruses In 1948 DALLDORF AND SICKLES (1949) isolated a virus pathogenic for newborn mice It was WP strain which they considered to be an independent immunologic type A7 Several strains of this type were isolated in 1953-1954 and studied in mice and in tissue culture in Sweden (JOHNSSON, 1956, SVEDMYR ET AL, 1956) These viruses were noted to adapt with difficulty to tissue culture However SVEDMYR, MELEN, KJELLÉN (1956) in collaboration with JOHNSSON (1956) isolated two A7 strains from stools on direct inoculation of tissue cultures, including one strain which was also isolated in suckling white mice Tests in adult cotton rats and monkeys with materials from which A7 virus had been isolated in mice or tissue cultures, were not done, as far as we know, until recently

In 1952 in the course of laboratory tests on cases of paralytic poliomyelitis from a small outbreak (260 cases) in the town of Karaganda, Kazakhstan, we isolated three peculiar strains of virus Two of these strains, AB IV and GZ-IV, were isolated by direct inoculation of monkeys with antibiotic treated stool suspension from paralytic patients, and were passed serially in monkeys The third strain, MK-IV, was isolated both in adult cotton rats and suckling white mice also inoculated with stool suspension from paralytic patients and was later adapted to monkeys

Both patients from whom the AB IV strain was isolated died of bulbo spinal poliomyelitis Tests in the monkeys, with pooled stool sample collected from these two patients on the first and sixth day after onset, did not result in isolation of poliomyelitis virus of any of the three types, but yielded the paralytogenic AB IV strain which was then propagated serially in monkeys This suggested that the isolated virus was the cause of fatal paralytic poliomyelitis in both or at least in one of these patients In another case of virus isolation (MK IV strain) convalescent serum from patient K contained high titers of homologous antibodies neutralizing MK virus Sera from other convalescents and gamma globulin from some regions of the U S S R also contained antibodies which neutralized Karaganda strains The strains were similar antigenically, but at the same time sharply different from type 1, 2 and 3 polioviruses and from many other neurotropic viruses

water, centrifuged at 3000 rpm for 30 min Supernatant fluid was removed and centrifuged at 12,000 rpm or 3000 rpm The second supernatant fluid was treated [illegible] streptomycin (250 units/ml) for an hour at [illegible]

[illegible]

guinea pigs, white rats, steppe [illegible]

cub were also inoculated with AB IV strain (with negative results)

[illegible] For virus isolation monkeys were injected over a [illegible] [illegible]onally 8–10 ml [illegible] ibiotics For [illegible] drawn with pincers and removed to the [illegible] al was introduced behind the tonsillar pillar of the opposite side, 0 5 ml into each tonsil [illegible] also received daily nose instillations of 2–3 ml of [illegible] [illegible]culated [illegible] d daily, [illegible] l during

Stool samples brought to [illegible] urs and tested by L L MIRONOVA in adult and suckling cotton rats and adult and suckling

[illegible] monkey [illegible] Adult [illegible] d [illegible] negative

MK strain was isolated from pooled stool sample of patients M and K Tests with other stool samples in rodents, and with brain and cord from a fatal case in monkeys were negative

Fecal material sent to Sukhumi was tested only in monkey experiments A total of 5 monkeys were inoculated The first animal received pooled faeces from patients A and B and yielded the AB strain

The second monkey received pooled faeces from 5 children with paralytic poliomyelitis, and strain "FD CHEMPO" was isolated

The third monkey received stool samples collected in the pre paralytic phase from patient Iv, and the "Iv" strain was isolated

The strains "FD CHEMPO" and "Iv" were identified in cross immunity tests in monkeys, and subsequently in cross-neutralization tests in tissue culture as type 1 poliovirus strains

Two more monkeys remained normal One of them was inoculated with pooled stools from three patients collected 2 weeks after onset, and the second monkey was inoculated with stool from a mother whose baby was ill

a definite association of Karaganda virus isolates with human paralytic poliomyelitis the first clinical confirmation outside the U S S R was presented in a report by RANZENHOFFER, DIZON, LIPTON AND STEIGMAN (1958) They reported the isolation of a virus regarded as Coxsackie A7 from a 3 year old boy admitted July 9 1956 to a Louisville hospital with a clinical picture of paralytic poliomyelitis (right leg paralysis demanding orthopedic treatment, and facial palsy) The virus was isolated twice in suckling white mice from two stool samples collected 1 and 2 days after admission Tests with the same samples in monkey kidney cell cultures were negative Serum samples collected 1 day, 2 weeks and 1 year after onset did not possess antibodies to the 3 standard poliomyelitis viruses on repeated tests in tissue culture At the same time, in convalescence the patient developed antibodies neutralizing Coxsackie A7 virus in titer 1 630 The authors concluded that they had confirmed the findings of the Russian workers.

VOROSHILOVA, TOLSKAYA, GAIDUCHENJA, BROMBERG AND LESHINSKAYA (1958) *in a study of paralytic poliomyelitis cases in the* town of Karaganda isolated 7 virus strains which were neutralized by antisera against the AB IV and also against a Coxsackie A7 strain, as well as by sera from patients collected in early convalescence This investigation is not yet completed, but sera of at least 3 of the con valescents did not contain antibodies for type 1, 2 and 3 poliomyelitis viruses

Thus data are accumulating in favor of an etiological role for AB IV Coxsackie A7 group of viruses in a human paralytic disease *clinically indistinguishable from paralytic poliomyelitis*

This paper presents a summary and an analysis of the data obtained in investigations *of the AB IV Coxsackie A7 group* of viruses

Isolation and Passage of Virus Strains

In 1952 a poliomyelitis outbreak occurred in the town of Karaganda Kazakhstan *Our laboratory carried out virological investigations* for isolating [illegible] and for typing the causative virus [illegible]

[illegible]

lytic patients and one brain and cord from a fatal case [illegible] to Moscow The other one consisting of 12 stool samples was brought to *Sukhumi* Medico Biological Station of the Academy of Medical Sciences of the U S S R.

Preparation Brain and cord from a fatal case were prepared as 10% suspensions in sterile saline Faeces were made into 20% suspensions in sterile distilled

lumbar cord, medulla, pons, midbrain, subcortex, cerebellum and different areas of cortex were put in 70% alcohol for Nissl staining, and the remaining material from CNS and viscera was fixed in 10% formalin

Histologic examination of the central nervous system of monkey No 1255 (ROBINSON, VOROSHILOVA, 1953) showed most marked lesions in spinal cord and medulla There was considerable disappearance of anterior horn nerve cells, degenerative changes in many remaining cells, neuronophagial nodules, diffuse tissue infiltration with inflammatory elements, and perivascular infiltrates mainly in lumbar cord

In the medulla the most marked lesions were in Deiters' nucleus, reticular substance and hypoglossal nerve nucleus

Disappearance of nerve cells, neuronophagial nodules, diffuse and perivascular infiltration were found in the thalamus and hypothalamus and in the nuclei of the cerebellum In the motor cortex there were small foci in the 5th layer Small lesions were found in globus pallidus and substantia nigra

On November 13, 1952, cord suspension from monkey No 1255 was inoculated into 7 cotton rats, of which 5 were found dead on the 6th postinoculation day

Experiments on the adaptation of AB IV strain to small laboratory animals were continued in Moscow in February 1953 (Fig 1) After cord suspension from monkey No 1255 was inoculated into suckling white mice 10 of 16 animals developed disease Several sucklings developed paresis of extremities, but in the majority of animals the clinical picture was indefinite After three brain passages in suckling mice, cotton rats were injected One of 3 cotton rats developed paralysis of hind legs on the 15th postinoculation day With this rat's brain suspension 17 suckling cotton rats were inoculated, 8 of which developed disease on the 7th–16th postinoculation day with paralysis of the hind legs On further passages in suckling cotton rats the incubation period became shorter and stabilized Now 100% of animals develop disease in 4 days after inoculation and die

After 14 passages in suckling cotton rats, on September 14, 1953, a 2 year old male rhesus monkey, No 103, was inoculated intracerebrally On the 6th postinoculation day the temperature rose, the monkey became restless On the 3rd day of illness the temperature fell and complete paralysis of the right leg developed, the cerebro-spinal fluid showed a pleocytosis of 311 cells per cmm Subsequently the monkey developed muscle atrophy and right knee joint contracture

After 25 passages in suckling cotton rats, on January 3, 1955, rhesus monkey No 21 was inoculated intramuscularly with suckling cotton rat brain suspension On the 7th postinoculation day the temperature rose to 40 4° C, and next day the monkey was found in a very severe condition, with tetrapareses paralysis of back and neck muscles, and left-sided facial paresis The cerebrospinal fluid had 411 cells per cmm (89% lymphocytes, 11% polymorphonuclear leucocytes) In this line of the AB-IV strain (Fig 1, left) the virus was carried

Thus from 5 positive groups of specimens from children with paralytic poliomyelitis in Karaganda two type 1 poliovirus strains were isolated and also three additional strains which subsequently were regarded as type IV poliomyelitis virus

AB IV strain isolation Patient A, 16 months old, became ill at 5 o'clock a m, September 17, 1952 He was unconscious, and stopped moving his legs and arms At 9 o'clock, on admission to a hospital there were no active movements in the upper and lower extremities Tendon reflexes were absent, nuchal muscles rigid, respiration difficult and intermittent His condition rapidly deteriorated and at 1 o'clock in the afternoon he died with evidence of respiratory center paralysis At autopsy softening of the cervical and lumbar cord, and pin point hemorrhages in the brain substance were noticed The picture of acute poliomyelitis was confirmed histologically

Patient B, 31 months old, became ill on September 12, 1952 The temperature rose to 39 5° C, and there was profuse sweating On September 15 movements of arms and legs stopped On September 16, on admission, the temperature was 39 2° C, voice very weak, cough non productive, mild facial palsy was present on the right side In the upper extremities only finger movements were present, in the lower extremities no movement at all Muscles of both upper and lower extremities were markedly hypotonic Tendon and periosteal reflexes in upper and lower extremities and also abdominal reflexes were absent Nuchal muscles were stiff, bilateral positive Lasègue's sign Thoracic muscles did not take part in the act of respiration On the following day the child's condition aggravated Finger movements disappeared Respiration became intermittent and shallow, heart sounds muffled, and on September 23, the girl died with symptoms of collapse and respiratory disturbance

At autopsy areas of softening were found in cervical, thoracic and lumbar cord, and capillary hemorrhages in the brain substance Histologically the diagnosis of acute bulbo spinal poliomyelitis was confirmed

Stool specimens were taken from both patients on September 17 (1st and 6th day after onset, respectively), and on September 18 they were brought to *Sukhumi*

The fecal material was pooled, treated and inoculated repeatedly into the macacus hybrid *(M rhesus ~ M nemestrinus)* No 1255 from 23th to 27th of October

Five days after the first inoculation the animal's temperature rose and reached the maximum of 40 3° C on the 7th day On the 10th postinoculation day paresis of the right leg developed

On the next day the right leg was completely paralysed, also paresis of the left leg developed in proximal parts On the 12th day no change in the animal's condition was observed It could move about the cage on the *arms, the legs* passively dragging on the floor On November 1, a lumbar puncture was done The cerebro-spinal fluid came under raised pressure, was clear and colorless with a pleocytosis of 212 cells per cmm (lymphocytes 92%, neutrophilic *leucocytes* 8%) The second lumbar puncture on November 4 showed a pleocytosis of 193 cells per cmm, all lymphocytes

On the 12th post inoculation day, the monkey was sacrificed *Pieces of* brain and cord were put in 50% glycerol Small pieces from cervical, thoracic and

with cord suspension from monkey No 1255 On the 4th day the monkey's temperature rose and persisted high for 3 days On the 5th day decreased tone of muscles of the upper extremities, especially on the right side, was noted The next day both arms were paralysed On November 9, the cerebro-spinal fluid showed a pleocytosis of 315 cells per cmm, predominantly lymphocytes

Simultaneously with monkey No 1523, 11 suckling cotton rats were inoculated with the cord suspension Nine of them were paralysed 4–6 days after inoculation

Third passage of AB-IV strain in monkeys rhesus No 1545, 9 years old, was inoculated with cord suspension from rhesus No 1523 on November 10, 1953 Illness developed 5 days after inoculation, and by the 10th day progressed to paralysis of the right leg and paresis of the left leg, when the monkey was sacrificed The cell count was 218 cells per cmm of spinal fluid

Simultaneously with monkey No 1545, 4 suckling cotton rats were inoculated, 3 of which were paralysed 4 days later The line of suckling cotton rat brain passages "AB 1523" has been regularly used in all our experiments since April 1956 At present 63 passages have been carried out in suckling cotton rats

On April 18, 1954, a 4th successful passage was made with 10% cord suspension of rhesus No 1545 after 5 months' storage in glycerol (see Fig 1), both into a monkey and into cotton rats

In 1956 attempts were made to continue the direct "monkey line" of the AB-IV strain However inoculation of monkeys with material from rhesus No 1563 (4th passage) and No 1523 (2nd passage) did not produce disease in monkeys, suckling cotton rats and suckling white mice, and histological examination of CNS of inoculated monkeys gave negative results

On November 29, 1956, rhesus monkey No 55 was inoculated intracerebrally, intraspinally and intramuscularly with 10% cervical cord suspension from rhesus No 1545 after 3 years' storage in glycerol The monkey did not become ill but histological examination of spinal cord and medulla revealed lesions characteristic for poliomyelitis

A more successful result was achieved in 1957 On July 3, 1957, rhesus monkey No 348 was inoculated intracerebrally, intraspinally and intramuscularly with 20% suspension of lower segments of thoracic cord At the same time it received intramuscularly 200 mg cortisone acetate and 300,000 units penicillin 5 days after inoculation the monkey was languid, there were tremors of arms and legs The cerebro-spinal fluid contained 87 cells per cmm On the following day deep paresis of right leg and less manifested paresis of left leg and arm developed

Thus AB IV strain was carried through 4 brain passages in monkeys From each of the monkeys it was easily adapted to suckling cotton rats, in which it readily propagated

In the first attempts to adapt the strain to suckling white mice the symptoms were not very clear in the majority of cases After 3 suckling white mice passages and one adult cotton rat passage the "AB 1255" line was carried through 41 suckling cotton rat brain passages At the 14th and 25th passage level monkeys were inoculated which developed disease indistinguishable from experimental poliomyelitis

The "AB No 1523" (monkey Eder" line) was passed 63 times and

1 Suckling white mice paralysed 4th day

2 Adult cotton rats paralysed 15th day

3 Suckling cotton rats paralysed 5th day

4 Monkey paralysed

Fig. 1 Diagram of isolation of AB-IV strain from faeces of patients A and B

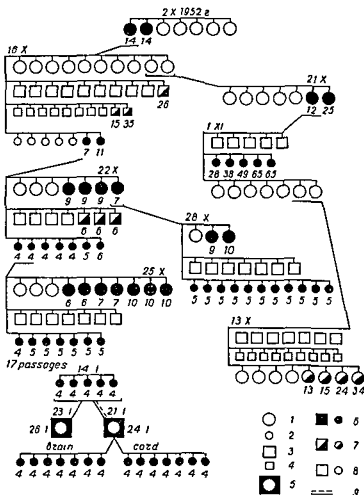


Fig 2 Diagram of isolation of "MK" strain in cotton rats (MIRONOVA, L. L., 1958)
1 Adult cotton rats, 2 Suckling cotton rats, 3 Adult white mice, 4 Suckling white mice, 5 Rhesus monkeys, 6 Animals paralysed, 7 Died without symptoms, 8 Remained well, 9 Muscles passaged

regularly produced paralytic disease in monkeys at the level of 31, 46 and 57 passages.

"MK-IV" strain isolation "MK-IV" strain was isolated in our laboratory by L.L. MIRONOVA (1954) from pooled stool samples from patients M and K.

Patient M, 15 months of age, fell acutely ill on August 9, 1952, with fever Four days after onset he could not stand on his feet On admission on August 15, he was very languid, could not sit and keep his head up Muscles of the legs, especially right, were hypotonic Flexion of knee joint was difficult, feet drop Knee and Achilles jerks absent, marked pains of radicular type On the 8th day after onset, 17 cells per cmm of spinal fluid, protein 0 45 gm% Then the patient's condition improved, he could sit and stand and even walk, when holding on to things Tendon reflexes appeared, except for the right Achilles The persistent sequela was the right foot drop which needed orthopaedic treatment

Patient K, 3½ years, fell acutely ill on August 9, 1952, with fever and pains in the legs The following day she could not stand up On admission, August 12, there was marked meningeal syndrome, with sharp radicular pains Muscles of the legs were hypotonic, movements limited, feet drop, reflexes completely absent The girl could not sit On the 6th day after onset the cerebro-spinal fluid showed 28 cells per cmm, protein 0 66 gm% In the course of treatment the girl could sit without help, but leg muscles remained hypotonic, atrophies developed, and tendon reflexes were not recovered

Stool samples from both patients were collected 6 days after onset, and brought by airplane to Moscow where they were kept frozen After centrifugation, antibiotics treatment, and bacteriological sterility tests the suspension was inoculated on October 2, 1952, into 7 cotton rats, 10 adult and 14 suckling white mice (Fig 2)

Two of the rats developed paralysis of both hind legs 14 days after inoculation The passages required to establish this line in mice, cotton rats, and monkeys are shown in Fig 2 After 17 passages in suckling cotton rats two rhesus monkeys were inoculated One received on January 23, 1953, 10% brain suspension, and the other 20% muscle suspension from suckling cotton rats Both monkeys developed illness and one of them died

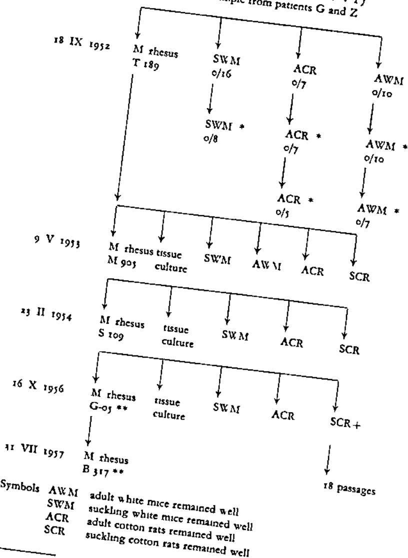
The second line of "MK" strain was established by passage in suckling white mice, which developed paralysis 7 days after receiving the human stool specimens Brain from three paralysed mice were negative but torso passage was more successful (see Fig 3) After the first carcass passage the inoculations were successful when done with brain and muscle suspensions from paralysed animals Guinea pigs, rabbits, suckling white rats were not susceptible to MK virus

In another passage series, the MK strain was isolated in the first blind passage done with four suckling mouse brains eleven days after initial inoculation Two out of eleven newborn white mice developed paralysis of one hind leg 37 and 43 days after inoculation, and passage with the carcass of one was successful in suckling cotton rats, but not in suckling white mice. In the next passage with brain suspension from the paralysed suckling rats paralysis developed in all inoculated suckling cotton rats and suckling white mice and one of six adult cotton rats

Thus, the MK strain was isolated in parallel experiments in adult cotton rats and in suckling white mice It proved to be most pathogenic for suckling cotton rats, and brought down monkeys after 17 cotton rat passages

Table I Diagram of isolation and passages of GZ IV strain (After IZELIS F G and ZHEVANDROVA V I)

Pooled stool sample from patients G and Z



* Passages from three animals which remained normal on the 7th day

Experiments done by GOLUBEVA N N

Fig 3 Diagram of "MK" strain isolation from faeces of two poliomyelitis patients in suckling white mice (MIRONOVA, L L, 1958)

1 Adult cotton rats, 2 Suckling cotton rats, 3 Adult white mice, 4 Suckling white mice, 5 Suckling white rats, 6 Guinea pigs, 7 Rabbits, 8 Animals paralysed, 9 Died without symptoms, 10 Torso passages

These findings should be considered in investigation of paralytic poliomyelitis cases not associated with type 1, 2 and 3 polioviruses For a complete study suckling mice, adult and suckling cotton rats and also monkeys should be inoculated

Pathogenicity for Laboratory Animals

Karaganda strains AB IV, MK IV and GZ IV are pathogenic for monkeys, (*Macacus rhesus*, *Macacus cynomolgus*), adult and suckling cotton rats (*Sigmodon hispidus hispidus*), suckling (but not adult) white mice, suckling steppe rodents (*Lagurus lagurus*), and Syrian hamsters (*Cricetus auratus*)

Multiple attempts to infect 7 to 12 gm mice with the AB IV strain both by combined routes (intracerebrally and intraperitoneally) and by separate intracerebral, intramuscular, intraperitoneal, intranasal and subcutaneous injections failed in all cases In addition three blind passages were done with each group of inoculated mice Pathogenicity for white mice was also tested during passages in suckling cotton rats In our experiments over 400 mice were inoculated

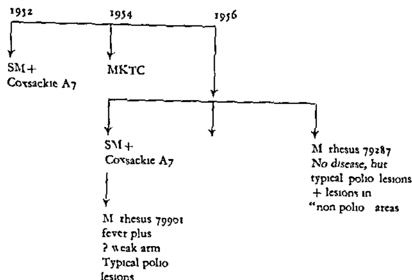
These data were confirmed in experiments by HABEL AND LOOMIS (1957) who failed to produce disease in adult mice by intracerebral or intraspinal inoculations with AB IV strain

Histologic examination of the central nervous system and muscles of adult white mice inoculated with AB IV strain did not show any lesions (ROBINSON, FROLOVA, SAVINOV AND SHEFTEL, 1958)

L L MIRONOVA (1954) never observed clinical disease in adult mice inoculated with Karaganda MK IV strain at different passage levels Fruitless also were 6 blind brain passages of MK IV strain in adult mice To study the possibility of virus survival in adult brains of mice, intracerebrally inoculated mice were killed at 1, 3, 6 9, 12, 15, 20 and 26 days Adult cotton rats were inoculated with brain and cord suspension Clinical disease was observed only in those cotton rats inoculated with suspensions from mice killed during the first three days after inoculation This was probably due to the survival of the virus in the mouse brain, but not true multiplication of the virus

In addition to serial brain passages, mice were inoculated intraperitoneally, subcutaneously and most frequently into the leg, to test for the absence of contamination with ectromelia virus Inoculated

Table III Diagram of isolation and passages of strain "Stool 6617" (After HABEL, K., and LOOMIS L., 1957)



white mice, with 3 additional passages (Fig 3, center) were negative The AB IV strain was readily isolated in monkey, its clinical and histological picture being completely indistinguishable from classical poliomyelitis Adaptation was quite successful in three species of animals, but from the very beginning suckling cotton rats were most susceptible

We had greatest difficulties in the interpretation of the results with the GZ IV strain The clinical picture in the first passage monkey was unlike poliomyelitis, perhaps because of inoculation trauma in the brain Suckling and adult white mice and adult cotton rats inoculated with material from patients did not develop *illness, either* on initial inoculation or on blind passages For some time persistent *attempts to adapt the GZ strain to rodents* were unsuccessful Its membership in this virus group was established only on the basis of immunity to AB-IV and MK IV strains in adult cotton rats immunized with CNS suspensions from monkey M 905 (GZ-IV strain), and on the basis of neutralization of AB IV and MK-IV strains with anti GZ IV sera Only after 3 passages in monkeys could a stable passage line in suckling cotton rats be established

Table IV (cont)

Monkey No	Material	Passage No	Dates of inoculation and autopsy	Route and dose	Incubation period (days)	Duration of fever (days)	Clinical picture
1317 *	AB-1545 1% cord suspension	4	18 IV 1954	IC 1 0	4	3	Left arm paralysis, atrophies 26 IV Cytosis 114
1302 **	AB-1545 1% cord suspension	4	18 IV 1954	IC 1 0	5	2	Left leg paralysis, atrophies, 27 IV Cytosis 68
55 ***	AB 1545 10% cord suspension	4	29 XI 20 XII 1956	IT 0 5 × 2 IS 0 5 IM 1 0	–	–	No clinical symptoms Typical poliomyelitis lesions in spinal cord and medulla oblongata
348 ***	AB-1545 20% cord suspension	4	3 VII 9 VII 1957	IT 0 5 × 2 IS 0 5 cortisone acetate 200 mg penicillin 300,000 units	3	2	At 5 days paralysis of right leg and less marked pareses of left leg and both arms Cytosis 260

* Previously was paralysed after inoculation with type I KRF I strain
** Previously was paralysed after inoculation with type III Leon strain
*** Experiments with participation of RALF, N M
**** All monkeys with cytosis had increased protein values (0 24–0 99‰)

IC – intracerebrally (frontal lobe)
IT – intrathalamically
IS – intraspinally
IP – intraperitoneally
IM – intramuscularly
IN – intranasally

Multiple attempts to infect adult mice with the third strain from Karaganda, GZ-IV, also gave negative results

Negative results were obtained when the AB-IV strain at different passage levels was given to 21 rabbits, 16 guinea pigs, 20 young white rats, 20 adult steppe rodents, one puppy, one fox cub IONESCU-MIHAIESTI (1957) failed to produce disease in young adult mice and hamsters

animals were held under observation for 1 to 3 months, but no signs of disease were noted.

Table IV Data on monkeys, inoculated with AB IV strain (direct monkey line)

Monkey No	Material	Passage No	Dates of inoculation and autopsy	Route and dose	Incubation period (days)	Duration of fever (days)	Clinical picture
1255 LASTIK	20% fecal *suspension* from patients A and B	1	23-27 X 5 XI 1952	IP 10×3 *tonsils* 0 5×3 IN 3×5	4	6	Right leg paralysis left leg paresis 1 XI Cytosis 212 4 XI Cytosis 193 Lymphoc 92%, leucoc 8% ****
1523 EDER	AB-M *rhesus 1255* 10% brain suspension	2	28 X 9 XI 1953	IC 1 5	3	3	Both arms paralysed, 9 XI Cytosis 315
1525	AB-1255 1% cord suspension	2	28 X 1953	IC 0 5	5	3	Pareses of leg muscles atrophies, contractures, 12 XI Cytosis 116
1508	AB 1255 1% cord suspension	2	28 X 1953	IC 0 5	6	3	Paralysis of left leg, paresis of right leg atrophies, contractures 12 XI Cytosis 101
1507	AB 1255 1% cord suspension	2	28 X 1953	IC 0 5	6	2	Left leg paresis, atrophies 12 XI Cytosis 219
1545 FULDA	AB-1255 10% cord suspension	3	10-20 XI 1953	IC 2 0	4	5	Paralysis of right leg, paresis of left leg 20 XI Cytosis 218
1563	AB-1545 10% cord suspension	4	18-27 IV 1954	IC 1 0	4	4	Paralysis of both legs 26 IV Cytosis 251

Table I Data on monkeys inoculated with AB IV strain after multiple passages in suckling cotton rats (Inoculation intracerebrally intraspinally or combined)

Monkey No	Material	Passage No	Dates of inoculation and autopsy	Route and dose	Incubation period (days)	Duration of fever (days)	Clinical picture
103	AB 1255 10% SCR brain suspension	Mon 1 SWM 3 ACR 1 SCR 14 *	12 IX 1953	IC 1 0	5	3	Right leg paralysed right side facial palsy 15 IX cytosis 109 lymph 72 9% neutr 25% monocyt 2 1%
1102	AB 1255 10% SCR brain suspension	Mon 1 SWM 3 ACR 1 SCR 15	21 27 IX 1954	IC 1 5	4	2	Paralysis of arms and legs back and neck muscles Aphony Respiratory failure 26 IX Cytosis 201 27 IX Cytosis 283
210 **	AB 1523 20% SCR brain suspension	Mon 2 SCR 31	22 28 III 1957	IC 0 5 × 2 IS 0 5 IM 1 0 Cortisone acetate 200 mg penicillin 300 000 un	[illegible]	2	Left leg paralysed
217 **	AB 1523 20% SCR brain suspension	Mon 2 SCR 31	4 18 IV 1957	IC 0 5 × 2 IM 1 0 Tonsils 1 0 × 2	3	3	Left leg muscles paresis

cases showed pleocytosis of 183 to 400 cells per cmm, lymphocytes predominating, sometimes with admixture of polymorphonuclear leucocytes The protein was 0 24 to 0 72 gm per cent

It is worth emphasizing one more peculiarity of poliomyelitis in these monkeys, namely the development of residual muscle atro

The MK IV strain (at 7, 10, 12, 13 and 23 passage levels) was negative in 16 rabbits, 16 guinea pigs, 5 suckling guinea pigs, 25 adult and 44 suckling white rats and 36 adult *L ligarus* (MIRONOVA, 1954 1958) POVALISHINA AND MIRONOVA (1958), also found an adult *L ligarus* to be resistant

Six rabbits, 6 guinea pigs, 20 white rats were inoculated with GZ IV strain with negative results, as were chick embryos when inoculated with the AB IV and MK IV strains

Pathogenicity for Monkeys
AB IV Strain

Experiments with the virus in "direct monkey line" Since 1952 the AB IV strain has been carried through 4 serial passages in monkeys The first monkey inoculated with fecal specimens from patients re presented an experimental hybrid of *Macacus mulattus* and *Macacus nemestrinus* Other animals were rhesus monkeys from 2 to 9 years of age Two of the experimental animals had been previously infected with type 1 and 3 strains poliovirus and survived paralytic disease produced by these strains Monkeys were inoculated as shown in Table IV

In the direct monkey passage line, ten of eleven animals developed disease with paralysis (see Table IV for details) This indicates a high paralytogenic activity of the virus in the CNS of monkeys One monkey in this series (Rhesus No 55), inoculated with M rhesus 1545 spinal cord kept in 50% glycerol for 3 years had no clinical signs of disease, but its spinal cord and medulla had typical poliomyelitis lesions Consequently, in monkey to monkey passages with AB IV strain all animals became infected

Monkeys which had paralyses ill as a result of type 1 and 3 infections had new paralyses in a different site after AB strain inocula tion Monkey No 1317 which had survived experimental polio-myelitis after type 1 KRF strain inoculation, with atrophic flaccid paralysis of both legs and right arm, developed paralysis of the left arm after inoculation with AB IV strain Monkey No 1302, which as a result of experimental poliomyelitis produced by type 3 Leon virus had had atrophies of the left arm and right leg, developed paralysis of the left leg after inoculation with AB IV strain

Examination of the cerebrospinal fluid from ten clinically manifest

Table V (cont.)

Monkey No	Material	Passage No.	Dates of inoculation and autopsy	Route and dose	Incubation period (days)	Duration of fever (days)	Clinical picture
18 M. cynomolgus	AB 1523 10% SCR brain suspension	Mon 2 SCR 57	11–16 VII 1958	ISo 2 × 3	5	–	Paresis of right leg, weak left leg and left arm Facial palsy Cytosis 202
15 M. cynomolgus	AB-1523 10% SCR brain suspension	Mon 2 SCR 57	11 VII	ISo 2 × 3 Cortisone acetate 200 mg	5	–	Weakness of left leg and left arm Cytosis 98

* Mon 1, SWM 3, ACR 1, SCR 14 – The strain had 1 passage in monkey, 3 passages in suckling white mice, 1 passage in adult cotton rats and 14 passages in suckling cotton rats

** Experiments with participation of Ralf, N M

muscles involved, in two of these monkeys contractures of knee joint and coxa also developed

Experiments in monkeys with AB-IV virus propagated in suckling cotton rats Twenty three monkeys (15 *Macaca mulatta* and 8 *Macaca cynomolgus*) were inoculated by various routes with suckling cotton rat brain suspension at different passage levels Intracerebral inoculation produced disease in 3 out of 3 monkeys, intraspinal in two out of two, intramuscular in 6 out of 10, intratonsillar in 3 out of 3, combined inoculation (in both thalami, intraspinally and intramuscularly) produced disease in five out of five animals (see Tables V and VI)

Beside direct inoculation of monkeys with brains from paralyzed suckling cotton rats, 4 monkeys were inoculated with CNS of monkeys brought down by cotton rat CNS These monkeys developed paralytic disease, with details shown in Table VII

Thus out of 27 monkeys inoculated with virus from the suckling rat passage line, only 4 monkeys did not become ill Those were the animals inoculated intramuscularly

The clinical picture of the disease in monkeys corresponded to experimental poliomyelitis Paralyses of all four extremities developed

Table V (cont)

Monkey No	Material	Passage No.	Dates of inoculation and autopsy	Route and dose	Incubation period (days)	Duration of fever (days)	Clinical picture
211 **	AB-1523 20% SCR brain suspension	Mon 2 SCR 31	4–9 IV 1957	ICo 5 × 2 IS 0 5 IM 1 0 Cortisone acetate 200 mg penicillin 300 000 un.	3	1	Paralysis of left leg, paresis of right leg and both arms Left-side facial palsy Cytosis 179
182	AB-1523 10% SCR brain suspension	Mon 2 SCR 31	4–11 IV 1958	ITo 5 × 2 IS 0 5 IM 1 0 cortisone acetate 200 mg	4	3	Paralysis of right leg paresis of left leg left arm and left side facial palsy Cytosis 333
192	AB-1523 10% SCR brain suspension	Mon 2 SCR 46	4–8 IV 1958	ITo 5 × 2 IS IM 1 0 Cortisone acetate 200 mg	3	1	Paresis of legs back muscle paralysis
10 M -cynomolgus	AB-1523 10% SCR brain suspension	Mon 2 SCR 57	11 16 VII 1958	ITo 5 × 2	5	–	Back and neck muscle paralysis, paresis of proximal parts of both legs decrease in power and tone of left arm diaphragm paralysis Cytosis 107

phies and contractures For example, five monkeys which were not sacrificed in the acute stage had residual paralysis and atrophies of the

Table VI (cont)

Monkey No.	Material	Passage No.	Dates of inoculation and autopsy	Route and dose	Incubation period (days)	Duration of fever (days)	Clinical picture
1371 M-cynomolgus	AB-1255 10% SCR brain suspension	Mon 1 SWM 3 ACR 1 SCR 15	21-22 23 IX 1954	Tonsils 1 0×3	3	–	Paresis of left arm and right leg 25 IX Cytosis 411 28 IX Cytosis 348 2 X Cytosis 96
2 M-cynomolgus	AB-1523 10% SCR brain suspension	Mon 2 SCR 57	11 16 VII 1958	Tonsils 1 0×2	5	–	Paresis of both legs back muscle paralysis, right side facial palsy hypoglossal nerve paresis, forced horizontal movement of eye balls Weak right arm 16 VII Cytosis 326
7 M-cynomolgus	AB 1523 10% SCR brain suspension	Mon 2 SCR 57	11 VII 1958	Tonsils 1 0×2 cortisone acetate 200 mg	5		Right leg paresis 16 VII Cytosis 271

* Mon 1 SWM 3 ACR 1 SCR 16 The strain had one passage in monkeys 3 passages in suckling white mice 1 passage in adult cotton rats and 16 passages in suckling cotton rats

(in 5 of 23 sick monkeys), of three extremities (5/23), of two extremities (6/23), or of one extremity (7/23) In addition in four monkeys there was involvement of back muscles, in 2 of neck muscles, in 5 of facial muscles and two had respiratory distress

In 5 of 6 intramuscularly inoculated monkeys, paralysis developed in the right leg into which the virus was introduced, or in both legs The sixth monkey, No 21, developed disease during menstruation and was very severely ill with tetrapareses, back and neck musculature paralysis and left facial palsy

Table VI Data on monkeys inoculated with AB IV strain after multiple passages in suckling cotton rats (Inoculation into tonsils or intramuscularly)

Monkey No.	Material	Passage No	Dates of inoculation and autopsy	Route and dose	Incubation period (days)	Duration of fever (days)	Clinical picture
1488	AB 1255 10% SCR brain suspension	Mon 1 SWM 3 ACR 1 SCR 16 *	3 XI 1953	IM 2 0	7	1	Paresis of both legs
1480	AB-1255 10% SCR brain suspension	Mon 1 SWM 3 ACR 1 SCR 16	13 17 XI 1953	IM 2 0	7	1	Paresis of both legs 9 XI Cytosis 161
1680	AB 1255 10% SCR brain suspension	Mon 1 SWM 3 ACR 1 SCR 16	25 XI 1954	IM 2 0	5	1	Paralysis of right leg pares s of left leg 1 X Cytosis 82
21 M -cynomolgus	AB-1255 10% SCR brain suspension	Mon 1 SWM 3 ACR 1 SCR 25	3 –10 I 1955	IM 2 0	6	2	Tetrapareses back and neck muscle paralysis left side facial palsy Cytos s 411
11 M -cynomolgus	AB-1523 10% SCR brain suspension	Mon 2 SCR 57	11 18 VII 1958	IM 2 0	5	–	Paresis of right leg 18 VII Cytosis 115
13 M -cynomolgus	AB-1523 10% SCR brain suspension	Mon 2 SCR 57	11 18 VII 1958	IM 2 0 cortisone acetate 200 mg	6	–	Paresis of right leg Cytosis 71

baby monkeys of 2 to 4 months (3 cynomolgus, one rhesus and one baboon hamadryad), and in two young cynomolgus monkeys None developed illness After 3 daily treatments of the nasal mucosa with 3% zinc sulfate solution, the AB strain (10% suckling cotton rat brain suspension having 4 0–4 7 log ID_{50} for adult cotton rats) was given per os in 5 ml doses for 7 days, November 3–9, 1953, to baboon hamadryad No 1485 Rhesus No 1484 and cynomolgus No 1473, 1452 and 1481 received virus on November 3, 4 and 5 in 10^{-2} dilution and in the following four days in 10^{-3} dilution During the period of November 14–24 fecal samples were taken daily from the monkeys and tested in adult cotton rats with negative results in all instances Tests with sera taken one month after feeding showed antibodies at 1 8 level in cynomolgus monkeys No 1473 and 1452 On April 20–27, 1954 the second feeding of monkeys with 10% suckling cotton rat brain suspension was done During the period May 2–12 daily stool samples were taken and tested in cotton rats, again with negative results Before the second feeding serum antibodies, in titer 1 4, were found only in cynomolgus monkey No 1452 One month after feeding cynomolgus monkeys No 1452 and 1473 had antibody titers 1 32, rhesus monkey No 1484—1 16, baboon No 1485, 1 4 On September 21, 22 and 23, 1954, the monkeys were inoculated into tonsils (each received 3 injections of 1 0 ml 10% suckling cotton rat brain suspension) By that time cynomolgus monkeys No 1452 and 1473 had antibody titers of 1 16, and rhesus monkey No 1484 had 1 8 None of the animals became ill, and repeated examinations of the cerebro spinal fluid gave negative results At the same time control cynomolgus monkey No 1371 four days after inoculation had tremors, and on the following day pareses of the left arm and right leg developed Thus as a result of feeding with AB strain the monkeys developed antibodies and immunity to subsequent inoculation into tonsils These data correspond to the results of 1950–1952 experiments with type I poliovirus in which immunity developed after repeated oral administration of virus

Horstmann and Manuelidis (1958) fed the AB IV strain to two young chimpanzees in 5×10^7 MID_{50} dose No illness developed and no virus was detected in the feces, but after feeding both chimpanzees developed neutralizing antibodies The authors suggest that these data correspond to the results obtained by Melnick and Kaplan (1953) who had earlier fed Coxsackie viruses to chimpanzees

Habel and Loomis (1957) studied the monkey pathogenicity of

Table VII Data on monkeys inoculated with AB IV strain after multiple passages in suckling cotton rats and one monkey passage

Monkey No.	Material	Passage No.	Dates of inocu-lat on and autopsy	Route and dose	Incubat on period (days)	Duration of fever (days)	Clinical picture
216	AB-211 20% spinal cord suspension	Mon 2 SCR 31 Mon 1	13 -18 IV 1957	ICo 5 × 2 IS 0 5 IM 0 85	3	2	Paresis of right leg Cytosis 187
317	AB-211 10% spinal cord suspension	Mon 2 SCR 31 Mon 1	19 -25 VI 1957	ICo 5 × 2 IS 0 5 IM 1 0	3	1	Paralysis of back muscles and of right leg Deep pa reses of left leg and both arms
316	AB 211 10% spinal cord suspension	Mon 2 SCR 31 Mon 1	19 -25 VI 1957	ICo 5 × 2 IS 0 5 IM 1 0 Corti sone acetate 200 mg penicillin 300,000 un	5	2	Back muscle para lysis Deep pareses of legs and arms
54	AB-18 10% suspension thoracic spinal cord	Mon 2 SCR 57 Mon 1	19 VII	ICo 5 × 2 ISo 2 × 3	4	2	Paralysis of left leg, paresis of right leg tremor in arms when catching things

Mon 2, SCR 31, Mon 1 = The strain had 2 passages in monkeys 31 pas sages in suckling cotton rats and one more passage in monkeys

When the virus was introduced only into tonsils, one out of 3 monkeys had paresis of one upper and the opposite lower extremi ties, the second had paresis of both lower extremities, back muscles paralysis, right facial palsy, hypoglossal nerve paralysis and weakness of right leg, the third one had paresis of one leg

Feeding experiments with AB strains were done in unweaned

Table VIII Comparison of paralysis distribution in monkeys infected with AB IV strain and Russian strains of type 1 poliovirus

Involvement	AB-IV		Type I poliovirus	
	Ratio	%	Ratio	%
Paralysis or paresis				
1–2 extremities	22/34	65	68/98	69
3–4 extremities	10/34	29	19/98	20
Very severe with prostration and death	1/34	3	5/98	5
Non paralytic poliomyelitis	1/34	3	6/98	6
Involvement of				
Back muscles	6/34	18	21/98	21
Neck muscles	2/34	6	22/98	22
Facial palsy	6/34	18	14/98	14

oculation at a dose of 200 mg to nine monkeys On the contrary, under similar conditions of inoculation cynomolgus monkeys No 15 and No 7 receiving cortisone acetate had much milder disease than cynomolgus monkeys No 18 and No 2 which were not injected with cortisone

According to our data, the severity of the paralytic disease was less in monkeys infected with the AB IV strain compared to experimental poliomyelitis caused by type 1 and type 3 viruses Comparison of the distribution of paralysis in monkeys infected with the AB IV strain and Russian strains of type 1 poliovirus (Voroshilova 1956) showed (Table VIII) that while involvement of one or two extremities was observed with almost equal frequency in both groups, three and four extremities were more often paralysed in monkeys infected with AB-IV strain, but neck muscle paralysis in the latter group was relatively rare In both groups there were cases of facial palsy, and non paralytic cases diagnosed on the basis of changes in cerebrospinal fluid and CNS lesions Ten out of 13 convalescent monkeys had muscle atrophies of extremities, three had contractures In one monkey (No 1488) which became ill in 1953 four years later the right leg was found 3 cm shorter than the left one, and significant muscle atrophy was present Ionescu Mihaiesti et al (1957) observed relatively mild paralysis in rhesus monkeys, but very

10% suckling cotton rat brain suspension from the 24th passage of AB strain which we brought to them in 50% glycerol One rhesus monkey (73542) inoculated intracerebrally had slight temperature rise at 3-7 days and moderate general tremors on the 4th day Tremor persisted for a week At 28 days a blood sample was taken, and the monkey received 5 ml of 20% suckling white mice brain suspension from the 2nd passage At 35 days another blood sample was taken, and the monkey was sacrificed for histological examination The second monkey (72929) received 1 0 ml of stock virus intramuscularly into the right leg It had fever on the 3rd and tremor on the 4th day On the 5th day it had weakness of both legs, but no progress was seen on the following day and the monkey was sacrificed Suckling white mice inoculated with lumbar cord suspension from this monkey developed paralytic disease Five rhesus monkeys inoculated intra muscularly with the same emulsion had no clinical signs No attempts were done to carry out intracerebral monkey passages of the virus Histologic examination of both monkeys showed typical polio myelitis lesions, one monkey had lesions only in the spinal cord

HORSTMANN AND MANUELIDIS (1958) studied in detail the AB IV strain in monkeys The 24th passage of suckling cotton rat brain caused "poliomyelitis like" disease in about half of monkeys in oculated intracerebrally (0 3 ml in each thalamus), intramuscularly (2-5 ml) or intraspinally (0 1 ml) Nine serial passages were done with combined inoculation of monkeys intracerebrally, intraspinally, intra muscularly and subcutaneously In addition the monkeys received 3 daily injections of 300 mg of cortisone Monkey cord suspension from the first 6 passages was positive for suckling mice In each of the first five passages only one of two injected monkeys was ill, in the 6th passage 3 out of 4, in the 7th 4/4, in the 8th 3/6 and in the ninth 1/3 No significant increase in severity of disease or shortening of incuba tion period was observed Three out of 4 monkeys in the 7th passage developed most severe paralysis, but in the 8th passage the disease was much milder The course of illness in monkeys was similar to that seen in mild poliomyelitis However recovery from paralysis was more rapid than seen in poliovirus infections one monkey of the 7th passage had prostration with quadriplegia, but 48 hours later the monkey could move all four extremities and complete recovery soon followed

It should be noted that we did not observe a disease enhancing effect of cortisone acetate which we injected at the time of virus in

Table IX Virus distribution in monkeys paralysed by the AB-IV strain and by the type 2 poliovirus strain Neva

S ain	AB-IV s ra n							Type II "Neva" s ra n
Monkey No	M -rhesus					M -cynomolgus		
	1525	545	563	02	2	10	2	M rhesus No 17
Passage	Mon. 2	Mon. 4	Mon. 4	Mon. swm 3 scr scr 5	Mon. 1 swm 5 scr 1 scr 25	Mon 2 scr 17		MKTC 5
Route and dose	IC 1 5	IC 2 0	IC 0	IC 5	IM 2.0	IT 0.5×1	Tonsils 2.0	IM 1 0
Sp nal cord	*					**	**	
cervical	3 7	3 3	4 0	4 2	4 5	+	+	4 3
thoracic	3 7	3 0	4 2	3 5	4 0	+	+	4 3
lumbar	3 2	4 3	4 0	4 4	5 0	+	+	4 3
medulla	2 0	1 0	2 2	3 2	2 0	+	+	4 8
pons	+	+	+	+	+	+	+	4 3
midbrain	+	+	+	+	+	+	+	3 3
subcortex	+	+	+	+	+	—	+	—
motor cortex	+	+	+	2 0	+	+	+	2 8
frontal	±	o	±	±	±	±	±	o
parietal	±	o	±	±	±	±	±	o
temporal		+	±	±	—	±	±	o
occipital	o	o	±	—	o	±	±	4 3
cerebellum	±	±	o	±	±	o	±	—
spinal ganglia	o	o	o	±	o	o	±	o
heart	o	o	o	o	o	o	o	o
lungs	o	o	o	o	o	o	o	o
liver	o	o	o	—	o	o	o	o
spleen	o	o	o	o	o	o	o	o
kidney	o	o	o	o	o	o	o	+
adrenals	o	o	o	o	+	o	o	+
tongue	o	o	o	o	o	o	o	o
tons ls	o	o	o	o	+	o	o	o
inguinal lymph node	o	o	o	o	o	o	o	o
mesenteric lymph node	o	o	o	+	o	o	o	o
stomach wall	o	o	o	o	o	o	o	—
large intestine wall	o	—	o	o	o	o	o	o

severe, fatal paralytic disease in cynomolgus monkeys, with typica poliomyelitis lesions in their spinal cord

Distribution of the Virus in the Body of Monkeys Paralysed as a Result of Inoculation with AB IV Strain

The virus titer in the spinal cord of the first passage monkey, M rhesus No 1255, was log ID_{50} 3 5 in cotton rat titration More detailed studies of virus distribution in the brain and cord, viscera and nerves were made in rhesus monkeys No 1253 (2nd passage), No 1545 (3rd passage), No 1563 (4th passage), No 1102, No 21, and cynomolgus monkeys No 10 and No 2 inoculated by different routes and at different passage levels of the AB IV strain (Tables IV, V, VI)

The virus titer in the spinal cord ranged from log ID_{50} 3 0 to 5 0 It was approximately the same in cervical, thoracic and lumbar cord independently of paralysis localization In the medulla the average was 1–2 2, but the highest level (3 2) occured in rhesus No 1102 which died with extensive paralysis, aphony and respiratory failure This monkey also yielded the highest titer (2 0) for motor cortex (Table IX)

When adult cotton rats were inoculated with 10% suspension of tissue from pons, mid brain and motor cortex more than half of the animals were paralysed, while on inoculation with 10% suspensions of subcortex, frontal, parietal and temporal cortex and of cerebellum only individual animals developed paralysis

Virus was not found in spinal cord ganglia in 5 out of 7 cases In one case (rhesus No 21) the virus was found in tonsils and adrenals, in another (rhesus No 1102) in mesenteric lymph node

Virus was never found in the viscera, intestinal wall and contents (apart from contents of large intestines), muscles, nerves, blood, nasopharynx, stool and urine

These data indicate a marked neurotropism of AB IV strain quite comparable to that of polioviruses, but with a slightly more diffuse virus distribution in the cerebral cortex

Pathohistologic Lesions in the Central Nervous System of Monkeys Inoculated with AB IV Strain

On histological examination, ROBINSON, VOROSHILOVA AND MIRONOVA (1954) and later ROBINSON, FROLOVA, SAVINOV AND

nuclei, reticular substance, nuclei of facial and hypoglossal nerves), and also in roof nuclei

Inflammatory lesions were represented by focal cell accumulation in the form of neuronophagic nodules, diffuse infiltrates with inflammatory elements, and also perivascular infiltrates Dependent upon the disease duration was the type of infiltrates leucocytic in the early phase, it became polyblastic microglial, and in later phases it was markedly astrocytic Examination of CNS obtained from a large number of monkeys inoculated with suckling cotton rat brain confirmed the original findings observed in the central nervous system of some monkeys inoculated in the direct monkey passage series We refer to certain peculiarities of the localization of lesions, as a rule not typical for experimental poliomyelitis In the spinal cord there was some extension of the pathologic process to posterior horns In the stem, lesions in some cases were more diffuse and extended to the pontile nuclei proper In the midbrain, not only were lesions seen in the substantia nigra, but small changes were also observed in red nuclei which are usually intact in poliomyelitis Non typical pathology was also found in the brain hemispheres where marked lesions were observed, not only in globus pallidus, which is sometimes involved in experimental poliomyelitis in monkeys, but also in putamen and more rarely in nucleus caudatus which as a rule are not affected in poliomyelitis

In the cerebral cortex, in addition to the lesions in the motor area which were regularly and significantly affected, there were lesions of neurons, formation of small loosely scattered neuronophagic nodules and perivascular infiltrates in the frontal, parietal, medullar and less regularly in the temporal cortex There was rather marked increased permeability of vascular walls and inflammatory changes in the pia mater No lesions were found in the viscera, muscles and brown fat

Horstmann and Manuelidis (1958) carried out a detailed serial histologic examination of the CNS of 13 monkeys inoculated with the AB-IV strain These authors also found that, as in poliomyelitis, the areas of the CNS most involved were the spinal cord, medulla, cerebellar nuclei, pons, motor cortex, thalamus and hypothalamus All animals showed lesions in these areas In addition, in most monkeys there were also lesions in the putamen, globus pallidus, caudate nucleus, and the cerebral cortex, including parietal and occipital lobes In some the involvement of the motor cortex was more marked than that in the medulla The lesions in the thoracic region of the spinal cord were less pronounced than in the cervical and lumbar areas In the cerebellar cortex occasional lesions were present in the vermis and very rarely in the cerebellar hemispheres Slight lesions which consisted only of perivascular infiltrates with occasional glial nodules were found in the frontal, parietal, temporal and occipital lobes of the cortex

SHEFTEL (1958) found most marked lesions in the spinal cord and medulla in all cases There was significant disappearance of anterior horn nerve cells in the gray matter of the cord, and in the medulla disappearance of nerve cells in typical poliomyelitis areas (in Deiters'

Table IX (cont)

Strain	*AB-IV strain*							*Type II "Neva" strain*
Monkey No	M -rhesus					M -cynomolgus		M rhesus No. 17
	1523	1545	1565	2102	21	10	2	
Passage	Mon 2	Mon 5	Mon 4	Mon 1 swm 5 acr 1 acr 15	Mon 1 swm 3 acr 1 acr 25	Mon. 2 acr 57		MKTC 5
Route and dose	IC 2 5	IC 2 0	IC 1 0	IC 2 5	IM 2 0	IT 0.5×2	Tonsils 2 0	IM 1 0
small intestine wall	o	o	o	o	o	—	—	—
large intestine contents	o	o	o	o	o	—	—	—
small intestine contents	o	o	o	o	o	+	+	—
urinary bladder	o	o	o	o	o	o	o	—
diaphragm	o	o	o	o	o	o	o	o
biceps muscle	o	o	o	o	o	o	o	o
triceps muscle	o	o	o	o	o	o	o	o
quadriceps muscle	o	o	o	o	o	o	o	o
calf muscle	o	o	o	o	o	o	o	o
sciatic nerve	o	o	o	o	o	o	o	—
blood	o	o	—	o	o	o	o	o
nasopharyngeal swab	—	—	—	o	o	o	o	—
stool	o	o	o	o	o	o	o	+
urine	o	—	—	o	o	o	o	o

* Log ACR PD_{50} = 5 7

** Titration of CNS tissues from cynomolgus monkeys No 10 and No 2 was not done

\+ On inoculation with 10% suspension most of the animals were paralysed

± On inoculation with 10% suspension only individual animals were paralysed

o All animals remained normal

— Not tested

Table XI Data on monkeys inoculated with GZ-IV strain
(After Izelis F G and Zhevandrova, V I)

Monkey No	Material	Passage No	Dates of inoculation and autopsy	Route and dose	Incubation period (days)	Duration of fever (days)	Clinical picture
T 189	Stools from patients G and Z	1	18 24 IX 1952	IC 1 0 IP 5 0 IN 3 0	Convulsions 1 hour after inoculation 21–22 IX convulsions		Tonic convulsions nystagmus general prostration, convulsions Died
M 905	GZ T 189 10% spinal cord *suspension*	2	9–15 V 1953	IC 1 2 IP 5 0 IN 4 0	6	–	Languor, nystagmus right side facial palsy, paresis *of left leg attacks* of tonic convulsions Died during such an attack Cytosis 1600
S 109	GZ M 905 10% spinal cord *suspension*	3	23-11 13 III *1954*	IC 1 2	11	–	Restless tremors Paralysis of left leg paresis of *right leg and right* arm Cytosis 291
G-05 *	GZ-S 109 10% cord suspension	4	16 X 1956	IT 0 5 × 2 IS 0 5 IM 3 0	4	–	Weak legs and right arm Cytosis 597 Neutroph 40% lymphocytes 58% monocytes 1% *macrophages* 1%
B-517 *	GZ-905 10% cord suspension	5	31 VII 6 VIII 1957	IT 0 5 × 2 IS 0 5			Paresis of left leg and arm, left side facial palsy

* Experiments done by Golubeva N N

The types of histologic lesions included changes of neurons manifested by different degrees of chromatolysis, perivascular and diffuse infiltrates and glial nodules In the spinal cord neuronal lesions were asymmetrical and confined to the anterior horns, although peri-

Table X Comparison of distribution and range of CNS lesions in monkeys infected with Russian AB IV strain of virus (Coxsackie A7), and poliomyelitis virus (from HORSTMAN, D M, and MANUELIDIS, E E, 1958)

	Russian AB–IV Strain of Virus			Poliomyelitis
	Animals with lesions		Range of lesions	Range of lesions
	No.	%		
Cord				
Cervical	13/13*	100	++ — ++++†	
Thoracic	11/11	100	+ — +++	++ — ++++
Lumbar	13/13	100	++ — ++++	
Medulla	12/12	100	++ — ++++	
Cerebral cortex				
Frontal	11/13	84	o — ++	±
Motor	13/13	100	++ — +++	+ — ++
Temporal	9/13	69	o — ++	o
Parietal	9/9	100	o — +	o
Occipital	9/13	69	o — +	o
Caudate nucleus	6/13	46	o — ++	o
Putamen	13/13	100	+ — ++	o
Globus pallidus	9/13	69	o — ++	+ — ++
Thalamus	13/13	100	++ — +++	+ — ++
Lateral geniculate body	2/10	20	+ — ++	o
Pons	13/13	100	++ — +++	±
Cerebellum				
Hemisphere	5/13	31	o — +	
Vermis	9/13	69	o — ++	
Roof nuclei	13/13	100	o — +++	+ — +++
Dentate nucleus	13/13	100	++ — +++	+ — ++

* Numerator indicates number with lesions, denominator, number examined

† Symbols o No lesions, + minimal inflammatory lesion or glial infiltration ++ moderate infiltration and minimal neuronal changes, +++ moderate infiltration and moderate neuronal changes, and ++++ severe infiltration and neuronal damage

cells in the spinal cord, medulla and dentate nucleus, loosely formed glial nodules with cell pycnosis and astrocyte proliferation

Table X compares the histological lesions in monkeys infected with AB IV strain and poliomyelitis virus HORSTMANN AND MANUELIDIS came to the conclusion that besides the marked lesions in areas typical for poliomyelitis, there were small inflammatory changes in frontal, temporal, parietal and occipital lobes of the cortex in 69–100% of animals The nucleus caudatus was affected in 6 out of 13 monkeys, putamen in all cases In 2 of 10 monkeys inflammatory lesions were found in the lateral geniculate body Extensive involvement of the motor cortex and dentate nucleus of the cerebellum was noted As a whole, the process was relatively milder, with less pronounced phagocytosis, than is usually seen in poliomyelitis

Monkey Pathogenicity of the GZ-IV Strain

GZ-IV strain had a total of 5 monkey passages The death of the first monkey No T 189 was at first considered nonspecific because 1 hour after inoculation it already had convulsions which were then observed during the next 4 days Despite that, its brain was put into glycerol and after 7 5 months' storage, the virus was successfully passed (see Table XI for details) The 3rd passage monkey, C-109, became ill after a longer incubation period and the disease was quite indistinguishable from typical experimental poliomyelitis, both clinically and histologically

Monkey Pathogenicity of the MK IV Strain

Three monkeys were inoculated with MK IV strain (MIRONOVA, 1954), as described in Table XII Three days after inoculation all the monkeys became restless, the fur was ruffled, there was marked general tremor, then paralysis and convulsions developed Stiff nuchal muscles and Kernig's sign were observed Two monkeys died during an attack of convulsions the third one was sacrificed in the state of prostration with flaccid paralysis of legs and right side facial palsy In all three cases the spinal cord was very severely involved, with marked disappearance of nerve cells and dense tissue infiltrates with polymorphonuclear leucocytes There was pronounced meningitis of

Table XII Data on monkeys inoculated with MK IV *strain* (After MIRONOVA, 1954)

Monkey No.	Material	Passage No	Dates of inoculation and autopsy	Route and dose	Incubation period (days)	Duration of fever (days)	Clinical picture
B-01	MK strain 10% SCR brain suspension	17	23-28 I 1953	IC 1 5 IP 10 0 IN 2 0	3	–	Restless ruffled fur, tremor of extremities then paralysis of leg muscles Right side facial palsy Attacks of convulsions 27 I Cytosis 53/3
1212	MK strain 10% SCR brain suspension	ACR 2 SCR 18	24 III 1953	IC 1 0 IM 4 0	3	–	General tremor fascicular muscle twitching Paresis of right arm back and neck muscles Attacks of convulsions
A 06	MK strain 20% SCR muscle suspension	ACR 2 SCR 17	21-24 I 1953	IC 1 5 IP 10 0 IN 2 0	3	–	Pendulum movement of eye balls Tremors Periodic attacs of convulsions Nystagmus Left side facial palsy Prostration

vascular infiltrates and glial nodules were also seen in the posterior horns The majority of the affected neurons in the spinal cord and other parts of the CNS became dissolved Neuronophagic nodules typical for poliomyelitis were also observed Acidophilic necrosis was found in nerve cells in the spinal cord, cerebellar nuclei and motor cortex

Perivascular infiltrates consisted mainly of lymphocytes Sometimes in the spinal cord, pons, cerebellar nuclei and motor cortex, diffuse infiltrates consisted of polymorpho nuclear leucocytes

Examination of the central nervous system of monkeys sacrificed 3 months after onset of severe illness showed disappearance of nerve

kindly sent to us from Prague by DRS ZACEK AND VANICKOVA, and two more monkeys, Nos 53 and 60, were inoculated with the WP strain kindly sent by DR JOHNSSON from Stockholm All 3 became ill (see Table XIII), and histological examination done by ROBINSON ET AL showed typical poliomyelitis lesions

Pathogenicity for Monkeys of Coxsackie A7 Virus Strains "Stool 6509" and "Stool 6617"

HABEL AND LOOMIS (1957) inoculated monkeys directly with stools from two nonparalytic poliomyelitis patients and with virus from suckling mice of the 1st passage Four out of 5 monkeys developed disease Table XIV compares poliomyelitis histologic lesions with lesions in monkeys inoculated with AB-IV virus and Coxsackie A7 strains It is seen that in a number of monkeys histological lesions were similar in localization to those of poliomyelitis, while in other monkeys in addition there were lesions of atypical localization

Pathogenicity for Adult Cotton Rats

The very first passages of the AB IV and MK-IV strains established their pathogenicity for adult cotton rats One of the lines of MK-IV strain was isolated on direct inoculation of adult cotton rats with faeces from paralyzed children In subsequent passages cotton rats regularly developed disease with paralysis after intracerebral inoculation The AB IV strain was carried through 11 serial adult cotton rat brain passages The virus titer in brain of paralysed cotton rats in titration experiments in adult cotton rats ranged from 10^{4} to 10^{-6}

After 4–6 days of incubation adult cotton rats develop paralysis (Table XV), more frequently of hind legs, indistinguishable from that produced by type 2 poliomyelitis viruses (ARMSTRONG, 1939) Paralysis of one hind leg was observed in 20% of animals, of both hind legs in 30%, one fore leg in 23%, both fore legs in 6% Paralysis of three extremities was seen in 6%, of all four in 15% Together with some paresis of leg muscles there was back muscle involvement in 23% Eight per cent of paralysed cotton rats survived and they had residual paralysis with muscle atrophies Adult cotton rats develop

monocytic leucocytic character Besides lesions in the motor cortex there were small inflammatory foci in the frontal, parietal and temporal lobes, and also, besides very marked lesions in dorsal parts of the stem, there were occasional small foci in the lower oliva and basal pontile nuclei

Thus in several monkeys infected with GZ-IV strain and in all 3 monkeys infected with MK-IV strain there were marked lesions in meninges, which was not typical for monkeys infected with AB IV strain

Pathogenicity for Monkeys of the Prototype WP Strain of Coxsackie A7 Virus

We observed disease in 3 monkeys inoculated with the prototype WP strain The first monkey was inoculated with carcass emulsion

Table XIII Data on monkeys inoculated with the prototype WP strain Coxsackie A7 virus

Monkey No.	Material	Passage No	Dates of inoculation and autopsy	Route and dose	Incubation period (days)	Duration of fever (days)	Clinical picture
28	A7 WP strain 20% carcass suspension		1 II 1957	IT 0 5 × 2 IS 0 5 IM 1 0	3	2	Decrease of muscle power weakness of left leg Cytosis 326
53	A7 10% suspension of carcass		19-23 VII 1 VIII	IT 0 5 × 2 IS 0 5 IM 1 0	5	2	High knee jerks Extension of re flexogenic zone Slight weakness of legs Cytosis 48
60	A7 10% suspension of carcass		19-23 VII 25 VII	IT 0 5 × 2 IS 0 5 IM 1 0	4	2	Decrease of power and tone of both legs, more of left No reflexes Then paralysis of left leg paresis of right leg and back muscles Cytosis 403

Dentate nucleus	+(+)	++	o	+	+++	++	+	+
Pontine nuclei	±			+	o	+	o	o
Corpus striatum								
Caudate	o	o	o	o	+	+	o	o
Putamen	o	o	o	o	+	+	+	o
Amygdala	+	o	o	±	+	+	±	o
Pallidum	+(++)	o	o	±	+	o	o	o
Olfactory centers	±							
Cerebral cortex								
Frontal	±	±	o	o	+	+	+	o
Pre-central	+(++)	++	o	++	+	++	+	o
Post-central	+	o	o	o	+	++	+	o
Parietal	o	o	o	o	+(+)	+(+)	+	o
Temporal	o	o	o	o	±	+	+	o
Occipital	o	o	o	o	±	+	±	o
Hippocampus	o	o	o	o	o	+	±	o
White matter	±	+	o	±	+(+)	+	+	o
Meninges	+(+)	+	o	+	+	+	o	o

* Symbols ++(++) = Range of ++ to 4 + o, no lesions, ±, absent or minimal lesions, +, mild or minimal inflammatory or glial infiltrations, ++, moderate infiltration and minimal neuronal damage, +++, moderate infiltration and moderate neuronal damage, ++++, moderate or severe infiltration and neuronal damage

† From Table II collection of D Bodian, Am J Hyg, vol 64 104, 1956, Technical Committee on Poliomyelitis Vaccine and Sub-committee on the Monkey Safety Test

Table XIV Degree of involvement and distribution of CNS lesions in monkeys infected with poliovirus, AB IV virus, and Coxsackie A7 (HABEL, K., and LOOMIS L., 1957)

Monkey No.	Par. and NP polio †	72929	73542	80951	79286	79287	79900	79901
Inoculated with	Poliovirus	AB-IV IM	AB-IV IC	Stool 6509 IC		S ool 6617 IC	SMP 1 Stool 6509 IC	SMP 1 Stool 6617 IC
Day after inoc.		6	35	7	55	35	37	37
Clinical signs		Tremors, fever weak legs	Tremors, fever	Fever weak arm		None	Fever ? tremors	Fever ? weak arm
Regions of CNS								
Spinal grey	++(++)	+++(+)	+	++(+)	++(+)	++(+)	+(+)	+(+)
Cranial nerve nuc	+(+++)	+++	o	+(+)	++	+	±	±
Retic. formation	+(+++)	+	o	+	+	±	o	o
Midbrain and subthal	+(+++)	+++	o	+(+)	++	+(+)	+	+
Substantia nigra	+(++)	++	o	++	++(+)	+	+	+
Hypothalamus	+(+)	+	o	++	+	++	±	o
Dorsal thalamus	+(++)	+	o	+	+	+	±	o
Cerebellar areas								
Cortex	+	o	o	o	±	o	o	o
Roof nuclei	+(+++)	++	o	++	+++	++	+	+

cord and brain (ROBINSON ET AL., 1958) In the spinal cord there were severe necrotic lesions of anterior horn motor cells, and their significant, sometimes complete, disappearance In place of dead cells there were accumulations of large numbers of polymorphonuclear leucocytes, polyblasts, histioid and macrophagic elements with admixture of microglial cells In neuronophagic nodules, a proliferation of astrocytes could be found Lesions were localized mainly in the anterior horns, but often also in the posterior horns of the gray substance of the spinal cord

In the medulla, pons, and midbrain, significant lesions were found in cranial nerve nuclei reticular substance, in nuclei of inner formation and dentate nucleus of cerebellum, and in red nucleus In the hemispheres the lesions were diffuse Degenerative changes of nerve cells in combination with mesenchymal reaction of vessel walls were found in the frontal, parietal, temporal cortex Ammon's horn, thalamus and subcortical nodules The histological picture in cotton rats inoculated with the AB IV strain was similar to that of experimental poliomyelitis observed in these animals after inoculation with Lansing strain (LILLY AND ARMSTRONG, 1940) but the lesions were more diffuse There were no lesions in muscles and viscera MIRONOVA (1954) found analogous lesions in adult cotton rats inoculated with MK-IV strain JOHNSSON AND LUNDMARK (1957) confirmed the above histopathological findings of the AB IV strain in cotton rats

Pathogenicity for Suckling Cotton Rats

In the very first passages of AB IV strain it became evident that of all rodents suckling cotton rats were most susceptible to it They developed disease 4–5 days after intracerebral inoculation The animals became languid, with ruffled fur, weakness of one or several extremities developed, soon followed by pareses or paralyses Frequently one of the first symptoms was back muscle weakness—the suckling could hardly turn over when on its back Duration of the disease did not usually exceed 8–12 hours In the terminal stage there was respiratory distress The fatality rate of sick animals was 100% Suckling cotton rats developed disease after intracerebral, subcutaneous, intramuscular, intraperitoneal and intranasal inoculation of virus containing material Table XV presents comparative data on

disease as a result of intracerebral, intraspinal, intramuscular and sub cutaneous inoculation Peripheral inoculation produces disease in cotton rats with less regularity

Within 7 to 9 days after inoculation of adult cotton rats, the MK IV strain (MIRONOVA, 1954) initially produced paralysis in about 65–70% of animals, *more frequently of hind legs, always with fatal* outcome Thus, 13 serial brain passages were done in 3-week old cotton rats In later passages morbidity and fatality was 100%, and incubation period shortened up to 6–7 days On inoculation of adult cotton rats the MK-IV strain had an intracerebral titer of log 5 0, intraperitoneally log 3 8, intramuscularly log 4 0, subcutaneously log 3 0, intranasally log 2 0 The GZ IV strain also regularly produces disease *in adult cotton rats The prototype Coxsackie A7 strain was* also found pathogenic for adult cotton rats and produced paralysis of extremities 4 to 7 days after inoculation (VOROSHILOVA, GOLUBEVA, 1957) Eight serial brain passages of this strain were done in adult cotton rats It should be said, however, that cotton rats paralyzed by intracerebral injection of the A7 strain, survive more often than do cotton rats inoculated with the AB, MK, and GZ strains

We studied the virus distribution in the body of paralysed adult cotton rats Blood and urine were collected, and then deeply anesthesized animals were washed through by circulation of saline The quantity of virus in the brain (including hemispheres, stem and cerebellum), cord, and organ suspensions was determined by adult cotton rat titrations

Two adult cotton rats paralyzed after intracerebral inoculation *with the AB strain had the virus in brain and cord in titer of* 10^{-4} to 10^{-5} There was no virus in muscles, heart, lungs, liver, spleen, kidneys, blood or urine Four adult cotton rats inoculated with 10% suspension of brown fat from one of the paralysed rats were all paralysed 6–9 days after inoculation despite the fact that other para lyzed rats showed no lesions in their brown fat Rats inoculated with 10^{-2} and 10^{-3} dilution of brown fat suspension failed to become ill *In the body of an adult cotton rat* paralysed as a result of the prototype A7 strain, the virus was found in brain and cord in 10^{-5} dilution, in muscles $10^{-4.5}$ Blood, urine, viscera and brown fat contained no virus

Histological examination of the central nervous system of paralyzed adult cotton rats inoculated with AB IV strain showed in all cases extensive diffuse inflammatory degenerative changes in the spinal

of the spinal cord In contrast to what was found in adult cotton rats, neuronophagia was very rarely seen Lesions were also found in the brain stem and hemispheres While in no case in monkeys and adult cotton rats were lesions in skeletal muscles found, suckling cotton rats almost always had diffuse focal lesions consisting of muscle swelling, disappearance of striations, globoid and granular destruction of individual muscle fibers

In areas of muscle destruction between fibers and in place of destroyed fibers there could be seen diffuse infiltrations with polyblasts and lymphoid cell elements After Goldman staining polymorphonuclear leucocytes could be seen among them In some cases such parenchymatous interstitial myositis involved almost all the muscles No specific lesions were found in the viscera and brown fat

ETINGOFF GERSHANOVITCH AND GOLUBEVA (1957) made biochemical investigation of brain homogenates of suckling cotton rats inoculated with AB-IV strain They studied anaerobic and aerobic glycolysis oxygen utilization succindehydrase aldolase and adenosintriphosphatase activity at the height of disease they observed a 20–30% decrease of aldolase and ATP activity when compared to control animals inoculated with normal brain suspension Inhibition of glycolysis was observed also for type 2 polio virus Beside the decrease of anaerobic and aerobic glycolysis in animals infected with the AB IV strain they observed a significant activation of the enzyme system concerned with the trans formation of succinic acid These findings correspond to the results obtained by RACKER (1942) in the study of biochemical changes of brain homogenates from animals infected with type 2 polio virus

Pathogenicity for Suckling White Mice

The AB IV strain was first adapted to suckling white mice in February 1953, however the clinical picture at that time was not quite clear, pareses developed only in rare animals and the incubation period was often as long as 10–14 days After adaptation to suckling cotton rats, the AB strain produced paralysis in suckling white mice with greater regularity But while the virus titer in suckling cotton rat brain was log 5 5–6 5 and in adult cotton rat 4 0–5 5, in the early passages in suckling white mice it never exceeded 2 5 The disease was similar to that described by *CASALS ET AL. (1951)* for type 2 poliovirus After 6 serial passages in suckling white mice, virus titers in the brain reached 6 0 Four or seven days after inoculation suckling

distribution of paralysis in cotton rats infected with the AB IV strain and Ovch-2 polio strain

Table XV Distribution of paralysis in suckling and adult cotton rats infected with AB IV and OVCH II (poliovirus) strains

Distribution of paralysis	Adult cotton rats		Suckling cotton rats	
	AB-IV	OVCH II	AB-IV	OVCH II
One hind leg	20 *	15	9	3
Both hind legs	30	30	55	21
One fore leg	23	27	6	2
Both fore legs	6	11	1	8
Three legs	6	9	3	24
Four legs	15	8	26	42
Back	23	23	86	78

* Per cent in each column was calculated on the basis of observations on 500 paralyzed animals

MK-IV and GZ-IV strains behaved similarly as regards patho genicity for suckling cotton rats MIRONOVA (1954) observed that one day old sucklings inoculated with MK strain may die 3–6 hours after languor and slight leg muscle weakness have appeared When titering the virus in suckling cotton rats, she observed simultaneous development of disease in animals inoculated with virus dilutions 10^{-1}, 10^{-2} and 10^{-3} With higher dilutions the incubation period was prolonged to 7–9 days

The following distribution of virus was found in bodies of sick suckling cotton rats inoculated with 18th passage MK-IV

Brain 6 7 *	*Spleen 4 1*
Cord 6 8	Intestine wall 4 4
Skeletal muscles 6 0	Blood 3 7
Liver 4 4	Stool 3 5
Lungs 4 3	Urine 3 3

Histological examination (ROBINSON ET AL, 1958) of the central nervous system of suckling cotton rats *inoculated with the AB IV* strain showed diffuse inflammatory infiltrates of gray matter tissue

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* Preliminary washing with saline through the *circulation was not done*

candles N, V and W They are resistant to ether for 24 hours, survive at room temperature for at least 74 days, and in frozen state at —20° C for at least 3 years The AB IV strain survives in 50% glycerol for at least 3 5 years, but there were cases when it lost its activity sooner, probably because inadequately purified glycerol was used, or because of low initial titer of the virus AB and MK strains are resistant to penicillin (500 units per ml) and streptomycin (250 units per ml)

Evidence for Homogenicity of Karaganda Strains

The dual characteristics of Karaganda strains—their neurotropic activity, marked for monkeys and adult cotton rats, and less so for suckling cotton rats and suckling white mice, and their myotropic activity for suckling cotton rats and white mice—raised the question of whether we were dealing with a mixture of two viruses—polio myelitis and Coxsackie viruses

Against this possibility was the regularity of the disease in the small laboratory animals, with typical clinical and histological pictures even after several passages in monkeys and the reverse—clinically manifest disease in monkeys even after 57 passages in suckling cotton rats Monkeys developed disease after inoculation not only with brain suspension but also with muscle suspension from paralyzed sucklings Therefore the virus having myotropic activity had at the same time neurotropic activity for monkeys Proof for the presence of only one virus with such complex characteristics in samples of different passage materials was obtained in cross neutralization tests with strain specific sera These tests were done in adult cotton rats, using 6 animals per dilution in the titrations

Horstmann et al (1958) neutralized the virus in monkey cord suspension 7th passage, with prototype Coxsackie A7 antiserum None of the 4 monkeys receiving the virus serum mixture became ill, and there were no histologic lesions in their brains Furthermore, virus isolated from monkey cord of the 6th passage was completely neutralized in suckling white mice by Coxsackie A7 serum Convalescent sera from two monkeys of the 7th passage of the AB IV strain neutralized 100 PFU of tissue culture adapted AB IV virus in dilutions 1 4 to 1 16 Sera against the polioviruses did not neutralize virus passed in adult cotton rats and in mice, and serum against AB IV virus did not neutralize the polioviruses

effects, though it remained viable in tissue culture for a long time (GOLUBEVA, N N, 1957)

Successful results were obtained by HABEL (1957) who adapted the AB-IV strain to tissue culture after 7 passages in monkey kidney cell cultures *After 33 tissue culture passages the virus titer was* 10^{6} per ml, and the cytopathogenic effect appeared in 24 hours In the 22nd passage, titer 10^{3}, the virus produced cytopathogenic effects in *tissue cultures of HeLa cells, human amniotic cells* and human fibroblasts Virus from the 8th TC passage was pathogenic for suckling white mice on intracerebral inoculation, but the virus from the 27th passage failed to cause disease in suckling white mice

Virus from the 23rd TC passage in Sabin's laboratory failed to produce paralysis in monkeys after intraspinal inoculation, but did yield poliomyelitis-like histological lesions

After two additional passages in monkey kidney cell cultures we inoculated the virus * into 50 suckling white mice, 50 adult and 65 suckling cotton rats, and 2 monkeys None of the rodents developed disease during 3 weeks of observation, but the monkeys developed mild tremors and weakness Histologic examination of the central nervous system of these monkeys showed lesions indistinguishable from those observed in mild poliomyelitis (ROBINSON ET AL, 1958)

Thus on long tissue culture propagation the AB IV strain lost its capacity to produce disease in small laboratory rodents, but preserved its neurotropism for the monkey central nervous system thus approaching more closely the character of true poliomyelitis viruses

Although there were general difficulties in propagation of Coxsackie A7 viruses in tissue culture it should be said that SVEDMYR, MELEN AND KJELLEN (1956) in collaboration with JOHNSSON (1956) studied two strains which were isolated from stools directly in tissue culture A third strain was isolated by them in suckling white mice and adapted to tissue culture

Properties of AB IV and MK-IV Strains

The AB-IV and MK-IV strains are readily filtrable through sterilizing asbestos pads, ST1 Seitz filter, and through Berkefeld

* We are indebted to DR K HABEL for his kindness in making this strain available to us

A1–10 (except for A7), and B1–5 viruses, and also against Mengo, encephalomyocarditis lymphocytic choriomeningitis, St Louis en cephalitis, types 1, 2 and 3 poliomyelitis viruses In adult or suckling white mice, monkey AB IV antiserum did not neutralize mouse encephalomyelitis TO, FA, GD VII viruses, Coxsackie A1–9, except for A7 In tissue cultures the same serum did not neutralize herpes, vaccinia, ECHO types 1–14 and types 1–3 polioviruses Complement fixation tests with AB IV serum gave negative results against types 1 and 2 polioviruses, Coxsackie A10, type 7 adenovirus, mumps virus Also, negative results were obtained in hemagglutination inhibition tests with AB IV serum and type 1 and 2 dengue, West Nile, yellow fever, Eastern and Western equine encephalomyelitis, Ilheus and St Louis encephalitis viruses

In experiments done by HABEL, as well as by JOHNSSON AND LUNDMARK, the AB IV virus was neutralized by Coxsackie A7 anti serum, and AB IV antiserum neutralized Coxsackie A7 virus in suckling white mice We obtained similar results in cross neutraliza tion tests in adult cotton rats (Table XVII)

Table XVII Cross neutralization tests

Serum \ Virus	AB-IV in adult cotton rats		A7 in adult cotton rats	
	4	16	4	16
AB IV	0/6	1/6	0/6	0/6
GZ IV	0 5	0/6	0/5	1/5
MK IV	1 6	2/6	0/6	1/5
A7	0/6	0/6	1/6	1/6
Ovch (Polio-II)	6/6	5/5	—	—

The virus dose was 100 ID_{50}

Surveys for Neutralizing Antibodies to AB-IV and MK-IV Strains

In 1952 we had before us the task of establishing the etiology of the Karaganda outbreak We did not try to collect sera on a large scale, because we did not have facilities to test them at that time However a small number of sera were taken in early convalescence, and in 1953 DR MIRONOVA made a special collection of sera Un fortunately many patients including those from whom virus strains

HABEL ET AL (1957) made 5 terminal dilution passages of the suckling and tissue culture virus lines, and even after this, the virus was completely neutralized by Coxsackie A7 antiserum Antisera prepared in adult mice against these two viruses obtained by terminal dilution passages cross neutralized each other both in suckling white mice and in tissue culture and also neutralized reference A7 virus in suckling white mice Virus from the 5th suckling white mice passage obtained by the terminal dilution method, were tested by SABIN for intraspinal monkey pathogenicity He reported that although the monkeys did not develop definite clinical paralysis, they had polio myelitis like lesions but distributed differently from those observed after infection with poliovirus

Thus on the basis of the above findings the AB IV strain may be considered as a single virus having poliomyelitis like properties and at the same time the ability to produce myositis in suckling white mice

Immunologic Characterization of the AB IV Strain

In cross immunity experiments in monkeys we showed the AB IV strain to differ from type 1 and type 3 poliomyelitis viruses (CHUMAKOV ET AL, 1956) Later these data were confirmed in neutralization tests with the AB strain and antisera for types 1, 2 and 3 polio viruses in adult cotton rats and in tissue culture AB IV strain anti serum did not neutralize types 1, 2 and 3 polioviruses

In cross immunity tests and cross neutralization tests in adult cotton rats the immunologic similarity of the AB IV, MK IV, and GZ IV strains was proved Cross immunity and cross neutralization tests run in parallel revealed the immunologic difference of Karaganda strains from type 2 poliomyelitis virus In addition the AB IV and MK-IV strains were not neutralized by sera from animals immunized with encephalomyocarditis (MM strain), mouse encephalomyelitis (GD VII strain), Russian tick borne encephalitis (No 4 and SOF strains), Japanese encephalitis (Nakayama strain), St Louis encephalitis, lymphocytic choriomeningitis (Yasakov strain), chorioencephalitis (BBBF strain), fixed rabies, louping ill, eastern and western equine encephalitis and equine meningoencephalomyelitis (MEL strain) viruses

According to HABEL ET AL. (1957) the AB IV virus was not neutralized in suckling white mice by immune sera against Coxsackie

Tests with convalescent sera from different towns of the Soviet Union showed antibodies in 28 of 51 cases All children were under 5 years It is important to say that two children had antibodies to AB virus in titers 1 64 and 1 512, but had no antibodies to the 3 types of polioviruses

HABEL ET AL (1957) tested sera of 29 healthy children of 1 to 15 years old from Washington The sera were used in 1/4 dilution against 100 ID_{50} AB IV virus propagated in suckling white mice (Table XIX) In each age group a significant number of sera were positive Tests with two lots of gamma globulin prepared in the U S A showed antibody titers 1 5 and 1 25

Table XIX AB IV antibodies in children of Washington D C (HABEL 1957)

Age (years)	Results of neutralization tests	
	Number tested	Number positive
1 5	10	6
5 10	12	7
10–15	7	3

Serum dilution 1 4 against 100 ID_{50} of virus in suckling white mice

Discussion and Conclusions

In 1952 investigations during an outbreak of poliomyelitis in the town of Karaganda suggested that viruses other than the 3 known types of polioviruses may be responsible for paralytic poliomyelitis These viruses were thought to belong to type IV poliomyelitis virus (CHUMAKOV, VOROSHILOVA ET AL, 1956)

In the experimental data presented Karaganda strains were shown regularly to produce in monkeys and cotton rats a picture of paralytic poliomyelitis usually, but not always, with several comparatively small peculiarities in the histopathology of the experimental infection The capacity to produce paralytic poliomyelitis in monkeys and cotton rats proved to be a constant characteristic of Karaganda strains and

had been isolated went for treatment to the southern resorts and we could not locate them

We did succeed in testing in adult cotton rats, sera from patient K (MK IV strain), and 32 more sera, 20 of which neutralized MK IV strain with an index over 100 The sera were tested in a constant dilution, 1 2, against different dilutions of the virus Patient K on the 12th day of illness and after 10 months had MK strain neutraliza tion indices of 1480 and 489, respectively These sera were not tested for neutralizing antibodies against the 3 types of polioviruses

In 1953 serologic tests were carried out with a constant serum dilution (1 2) and serial tenfold virus dilutions This method proved to be cumbersome and unsatisfactory for mass serological survey and subsequently we used one serum dilution 1 2 against 100 doses of virus in 0 025 ml (final dilution of serum 1 4 in 0 05 ml) After 1 hour of incubation at 37° C the mixture was introduced in doses of 0 05 ml intracerebrally to adult cotton rats, 6 animals per mixture Positive sera were then titrated in fourfold dilutions up to 1 256 At present serological tests are carried out in trypsinized kidney cell cultures or by the pH test

Tests with 18 lots of gamma globulin prepared in Moscow, Gorky, Odessa, Kharkov in all cases gave positive results in titers 1 4 to 1 128 All 30 pooled placenta sera, collected in different towns along the Volga, contained antibodies in titers of 1 4 to 1 64 Sera from healthy people of different ages were collected in Moscow, Riga, towns in the Volga basin, Lvov, Alma Ata, Karaganda and other towns of the Soviet Union (Table XVIII)

Table XVIII Summary of serological survey of AB IV antibodies in healthy people of different ages in the U S S R

Age groups	Results of neutralization tests		
	Tested	Positive	% positive
Up to 1 year	18	1	5
1 6	126	40	32
7 16	91	48	53
16 and over	110	89	80
Total	345	178	51

Table XX Comparison of behavior of polioviruses, viruses of group AB-IV – Coxsackie A7 and other Coxsackie A viruses

Species Characteristic	Polioviruses			Coxsackie A				
	1	3	2	AB-IV	A7	A1	A14	Others
Monkeys								
Paralytic poliomyelitis								
on inoculation into CNS	+	+	+	+	+	—	—	—
by peripheral routes	+	+	+	+	—	—	—	—
Characteristic changes in the cerebrospinal fluid	+	+	+	+	+			
Atrophies, contractures	+	+	+	+				
Virus in the CNS	+	+	+	+	+			
Histological lesions in the CNS of poliomyelitis type	+	+	+	+	+	—	+	—
Cotton rats								
Paralysis	—	—	+	+	+			
Histological lesions in the CNS of poliomyelitis type	—	—	+	+	+			
Histological lesions in muscles	—	—	—	—	—			
Suckling cotton rats								
Paralysis			+	+		+	+	+
Histological lesions of polio myelitis type			+	+				
Biochemical changes in the brain			+	+				
Virus in muscles			—	+	+			
Myositis			—	+	+			
Suckling white mice								
Paralysis	—	—	+	+	+			
Histological lesions in the CNS	—	—	+	+				
Virus in the brain	—	—	+	+	+	+	+	+
Virus in muscles	—	—	—	+	+	+	+	+
Myositis	—	—		+	+	+	+	+
Adult white mice								
Paralysis	±	±	+	—	—	+	+	—
Histological lesions in the CNS	±	±	+	—		+	+	—
Tissue culture	+	+	+	±	±	—	—	— (494)

one which did not decrease on serial propagation This capacity regularly manifests itself at different passage levels, with any virus-containing material-brains of monkey, adult and suckling cotton rat, and suckling white mouse, or muscles of the sucklings In addition these virus sources always possessed the property of producing diffuse myositis of cross striated muscles in suckling cotton rats and white mice In other words Karaganda strains were similar on the one hand to polioviruses—by their pathogenicity for monkeys and adult cotton rats, and on the other hand, to Coxsackie group A viruses, by their capacity to produce degenerative and inflammatory changes in muscles of suckling white mice, cotton rats and hamsters (Table XX) The dual character of the pathogenic properties of Karaganda strains for different species of animals (combination of neurotropism with myotropism) could create confusion in the classification of these strains First of all a question arose whether these strains were a mixture of one of the established poliovirus types and a Coxsackie group A virus This assumption did not prove to be correct and was rejected on the basis of exhaustive investigation in several laboratories

Owing to studies of JOHNSSON AND LUNDMARK (1957), HABEL AND LOOMIS (1957), HORSTMANN AND MANUELIDIS (1957, 1958), IONESCU-MIHAIESTI ET AL (1957), and also in our laboratory (1957, 1958) it was possible to discover close relationships existing between the Karaganda strain AB IV and Coxsackie A7 virus, as regards both cross antigenic reactions and pathogenicity for suckling white mice, cotton rats and hamsters Furthermore, these investigations revealed a heretofore unknown neurotropism of Coxsackie A7 virus and a paralytogenic capacity for monkeys Thus in the studies of the Karaganda strains, the known characteristics of Coxsackie A7 virus were considerably extended

These findings by themselves seem sufficient to us for raising the question of exclusion of A7 virus from the Coxsackie group of viruses The A7 strains differ from the Coxsackie viruses, for which absence of pathogenicity for monkeys was always considered pathognomonic

Are Karaganda strains AB IV, MK IV, GZ-IV identical to Coxsackie A7 virus? This question can not as yet be definitely answered, because further comparison of serological, epidemiological, clinical and experimental data is necessary, but there are several indications as to the existence not only of similar properties and

rats and monkeys (MK-IV, AB IV) All Karaganda strains and also virus isolated twice in Louisville (U S A) from a case of paralytic poliomyelitis (RANZENHOFFER ET AL , 1958) could not be isolated in tissue culture Consequently when at present routine methods of virus isolation in tissue cultures are used, not all cases of paralytic poliomyelitis" may be etiologically identified Those associated with "type IV poliomyelitis virus" or with neurotropic variants of Coxsackie A7 demand inoculation of monkeys and cotton rats However serological surveys can be made with the monkey kidney culture adapted variant of Karaganda AB IV virus Further search for new methods of detection of viruses similar to the Karaganda strains or to the A7 neurotropic variant will be necessary Even now there are *difficulties in virologic diagnosis of a number of paralytic poliomyelitis* cases, when negative results are obtained in tissue culture experiments or in animal experiments under conditions which seem optimal for the time of virus isolation and when paired sera fail to show a rise of antibodies to polioviruses (MELNICK, 1955, SCHMIDT AND LENNETTE, 1955) Perhaps all such "negative" cases of paralytic poliomyelitis must be tested for the development of antibody to AB IV virus and, when positive, further tested in animals

According to our data, and also those of HABEL AND LOOMIS (1957) human gamma globulin in the U S S R and in the U S A contains antibodies regularly neutralizing AB IV and Coxsackie A7 viruses which indicates their infectiousness both in Europe and in America

Are the present data sufficient to establish the etiologic role of AB IV-A7 group of viruses in human paralytic disease? For certain reasons we were not able in 1952 to present detailed serological data for the etiological role of the isolates in the paralytic diseases from which they were obtained The most important omission was the absence of serial tests for antibodies to the 3 types of polioviruses However, high titers of antibodies neutralizing AB IV and MK IV viruses were definitely shown in convalescents from paralytic disease in Karaganda, in cases from which MK IV strain and other similar strains were isolated (VOROSHILOVA ET AL , 1958)

In addition the circumstances of AB IV virus isolation in tests with stools from two patients collected on the 1st and 6th day of illness suggest with great probability the absence of any connection with type 1, 2 and 3 polioviruses, because only AB IV virus was isolated in monkeys It is improbable that in this phase, with the

relationships between them, but also of certain differences These are especially clear in comparison of the neurotropic intensity for monkeys and cotton rats Indeed, Karaganda strains were isolated from very severe cases of bulbo spinal poliomyelitis (including two fatal cases) under conditions excluding, in all probability, association of these cases with the recognized types of polioviruses The strains isolated in Karaganda preserved high paralytogenic activity for monkeys and cotton rats

Recently Coxsackie A7 viruses have been isolated in the U S A and in Sweden from cases of so called nonparalytic poliomyelitis (HABEL, SILVERBERG AND SHELOKOV, 1957) or from a case of transitory paralysis (KILBOURN AND GOLDFIELD, 1956), or from cases of aseptic meningitis and mild cases of meningoencephalitis (20 strains in 1954), (JOHNSSON, 1957) A7 strains were also isolated from healthy children, who were virus-carriers According to HABEL (1957) and DALLDORF (1957) Coxsackie A7 strains produce in monkeys only a very mild picture of paralytic disease or even asymptomatic infection, with histologic lesions in the spinal cord of a mild poliomyelitis type and less restricted in their distribution in the forebrain

Are quantitative differences in the intensity of human neuropathology connected also with qualitative species differences between these two groups of antigenically viruses similar? This question must as yet remain open Both groups of viruses are so close to each other that they should be considered together as representatives of one family of viruses despite their evident biologic variability On the one end of this family are strains with more manifest myotropism These strains are readily isolated in suckling white mice and do not appear to play any important role in human pathology, except for some cases of aseptic meningitis and meningoencephalitis (JOHNSSON, 1955, BERGLUND AND JOHNSSON, 1957) Their isolation in monkeys is possible but no extensive picture of paralytic poliomyelitis is produced On the other end are strains similar to Karaganda strains of type IV poliomyelitis virus which is originally highly pathogenic for monkeys and is adapted to suckling mice with difficulty The most striking representative is the GZ-IV strain, adapted to rodents only after three passages in monkeys Perhaps such strains can cause paralytic poliomyelitis in man which is not diagnosable in tissue culture and in suckling white mice For their isolation inoculation of monkeys is necessary, preferably cynomolgus monkeys, and, possibly, cotton rats The other strains of this group are more readily isolated in cotton

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extremely severe course of the acute disease (bulbar poliomyelitis), poliovirus would not have shown its activity if it had been the cause of the patient's death Unfortunately the brains from the fatal cases from which AB IV virus was isolated were not tested virologically (the CNS in these cases was examined only histologically) Analogous examples were presented from two other cases of isolation of Karaganda viruses from stools in the first days of "acute paralytic poliomyelitis"

More complete etiologic evidence was presented in a report by RANZENHOFFER ET AL (1958), who made two isolations of the same virus in suckling mice from samples collected one and two days after admission of a patient with acute paralytic poliomyelitis They showed the absence of any antibodies for the 3 polio types, but established rise of antibodies to the isolated homologous strain and to the proto type Coxsackie A7 strain

VOROSHILOVA ET AL (1958) discovered in Karaganda several cases of paralytic poliomyelitis in which AB IV virus was detected in the feces AB IV antibodies rose during the disease, but there were no polio antibodies in early convalescence At present our laboratory is continuing to work on the problem of the etiological role of the AB-IV-A7 group of viruses in human paralytic disease

In conclusion, we are *led to the inference that the group of* Karaganda AB IV strains and Coxsackie A7 strains should be regarded as an independent type IV poliomyelitis virus occupying an intermediate position between type 1, 2 and 3 poliomyelitis viruses and Coxsackie group A viruses This point of view should stimulate further accumulation of data on the etiology and immunology of "poliomyelitis", particularly in cases which are difficult to diagnose by the present tissue culture methods

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the intranuclear bodies to those described by TYZZER (1906) in skin lesions of varicella However, they stated that there was no evidence at that time to support the possibility of an unknown intracellular infection stimulating the cells to assume the unusual variation VON GLAHN AND PAPPENHEIMER (1925) reported the first case of cytomegalic inclusions in an adult who died of ulcerative colitis and hepatic abscess They recognized the similarity of the cellular changes to the inclusions described by LIPSCHUTZ (1921) in both spontaneous and experimental herpetic lesions, and for the first time the viral etiology of the disease was postulated

JACKSON (1920) described as parasites, the unusual large cells in the ducts of the salivary glands of guinea pigs However, it was not until 6 years later that the first experimental investigations of the disease in guinea pigs were reported by COLE AND KUTTNER (1926) Salivary gland material from old guinea pigs produced typical inclusion containing cells at the site of inoculation in young guinea pigs The material remained infective after passage through a Berkefeld N filter and was heat labile Serial passage of the virus was accomplished with subcutaneous inoculations of salivary gland material In the following years a variable incidence of cytomegalia was noted in the salivary glands of many other rodents (rats mice hamsters, and moles), in monkeys *(Cebus fatuellus)* and more recently in chimpanzees

The species specificity of the causal agent of the disease in guinea pigs was demonstrated by KUTTNER AND COLE (1926) and KUTTNER (1927) The presence of the characteristic inclusions in the salivary glands of the guinea pig, hamster and mouse were found to be associated with a transmissable filtrable virus but in each instance the virus proved to be entirely species specific (KUTTNER AND WANG, 1934 KUTTNER AND TUNG 1935)

The frequency of cytomegalic inclusions in the salivary glands of infants was emphasized by FARBER AND WOLBACH (1932) They found the incidence of the inclusions in the salivary glands to be 12 percent in 183 autopsies of infants and young children, regardless of the cause of death The incidence reported by others from widely separated parts of the world has varied from 8 to 32 percent (SEIFERT AND OEHME, 1957)

Reports of the pathology of the generalized form of the disease in infants, involving organs other than the salivary glands continued to appear in the literature but were not frequent until after 1950 There was a tendency to regard the peculiar inclusions as only

distinctive greatly enlarged cells But more than 30 years ago early investigations established the viral etiology of the disease in several rodents and the marked species specificity of the salivary gland viruses infecting these animals was demonstrated The similarity of the latent disease in the salivary glands of infants and young children to the salivary gland virus diseases of rodents indicated the viral etiology of the disease in man However the failure to recognize the clinical importance of the disease in man until recent years and the lack of an experimental animal susceptible to the human virus delayed further investigations The development of tissue culture techniques, providing a medium for the isolation and propagation of a species specific virus in homologous tissue, gave new impetus to study of the salivary gland viruses of both man and animals

Historical Resume

The greatly enlarged cells with intranuclear and cytoplasmic inclusions, now considered pathognomonic of salivary gland virus infection, were first described by JESIONEK AND KIOLEMENOGLOU (1904) They observed them in the kidneys, liver and lungs of a still born infant regarded as syphilitic In the same year RIBBERT (1904) reported identical large cells, occurring in the kidneys of a newborn infant, which he had observed 23 years earlier, and also in the parotid glands of two older infants Three years later LOWENSTEIN (1907) noted the occurrence of such cells in the ducts of the parotid glands of 4 of 30 infants Only a few other observations of these unusual cells in the organs of stillborn and young infants were recorded in the literature before 1921 The nature of the peculiar cellular changes was unexplained The possibility that they were protozoan parasites was discussed by RIBBERT and others SMITH AND WEIDMAN (1910 1911) classified them as a new species of endamoebae Others attributed the formation of these gigantic cells to syphilitic infection

GOODPASTURE AND TALBOT (1921) reported another instance of the presence of the characteristic cells in the organs of an infant, 6 weeks of age These authors regarded the peculiar cells as a type of cytomorphosis resulting from chronic inflammation and suggested the name ' Cytomegalia" They noted the resemblance of the cells found in infants to those reported by JACKSON (1920), in the salivary glands of guinea pigs, and also called attention to the similarity of

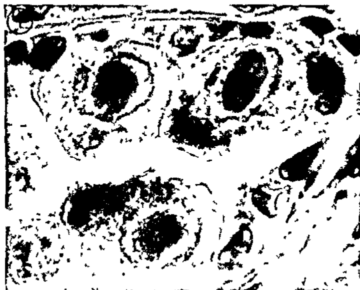


Fig. 1. Cytomegalic inclusion cells in a renal tubule. Cytoplasmic inclusions are concentrated in parts of cells nearest to lumen. Hematoxylin and eosin. 2000 ×.

periphery of the cell. The cytoplasm of the largest cells is frequently vacuolated and their contour irregular. The intranuclear inclusion may occur in a cell which does not contain cytoplasmic inclusions, but on the other hand the cytoplasmic inclusions never occur in a cell which contains a nucleus devoid of an inclusion. At times a nucleus cannot be seen in a cell containing cytoplasmic inclusions, either because the cell is so large that the nucleus is not in the plane of the section or because the cell has degenerated. The inclusions are usually found in epithelial cells but may occur in cells of the interstitial connective tissue and in vascular endothelium. Both the intranuclear and the cytoplasmic inclusions are Feulgen positive, the cytoplasmic inclusions staining less intensely than the intranuclear. The cytoplasmic, but not the intranuclear inclusions, react positively with periodic acid leukofuchsin (PAS stain).

The incidence of the intranuclear inclusions in the salivary glands of infants and young children has been reported variously as 8 percent to 32 percent in routine autopsies but only one record of these inclusions in the salivary glands of an adult has been found (MALOWITSCHKO AND

incidental findings in infants dying from other causes CAPPELL AND MCFARLANE (1947), SMITH AND VELIOS (1950) and WYATT AND ASSOCIATES (1950) compared the cases reported in the literature with additional ones which they had observed, emphasized the importance of the generalized disease in infants and pointed out the more frequent clinical and pathologic manifestations WYATT ET AL (1950) suggested that the characteristic cells might be found in the urinary sediment in some cases, and thus make possible a diagnosis during life

Since 1950 there has been an increasing interest in the human disease with many publications of case reports This current interest has been stimulated by the recognition of the importance of the disease in infants and by the possibility of confirming the clinical diagnosis by examination of urinary sediment, as well as by recent investigations of the viral agents of man and rodents

Generalized Salivary Gland Virus Disease in Man Clinical and Pathologic Manifestations

The distinctive morphologic feature of cytomegalic inclusion disease is the large inclusion bearing cell (Fig 1) Its nucleus is enlarged and the cytoplasm increased in amount The great size which may be attained by the cell (25 to 40 microns) and by the intranuclear inclusion (8 to 10 microns) is a strikingly unique characteristic The large intranuclear inclusion is surrounded by a clear halo which separates it from a distinct nuclear membrane containing one or more dense basophilic masses At times the nuclear membrane appears wrinkled or partially collapsed The shape of the inclusion usually corresponds to that of the nucleus in which it lies When stained with hematoxylin and eosin the intranuclear inclusion may be either acidophilic or basophilic However, the degree of basophilia of the inclusion is seldom as great as that of the nuclear membrane The inclusion may appear granular, or nodular, or uneven in density, at times the peripheral zone stains less intensely than the center Frequently, however, the inclusion appears homogenous Its outline may be either sharply defined or hazy The cytoplasm of the cells is acidophilic or amphophilic and frequently contains small basophilic bodies which vary in size and number The basophilic bodies measuring 2 to 4 microns in diameter are concentrated usually in one part of the cytoplasm and may be arranged in a concentric manner near the

tory reaction is most pronounced in subependymal tissues and in the olfactory tract (HAYMAKER ET AL 1954) Microcephaly, microgyria and hydrocephalus may result Aplasia of the cerebellum has been reported by DIEZEL (1954) Inclusions occur in ependymal and glial cells, less frequently in ganglion cells and endothelial and adventitial cells of blood vessels The foci of calcification are usually paraventricular but, in addition, may be more widespread

Epithelial cells of the proximal convoluted portions of the nephron are the site of predilection for the intranuclear inclusions in the kidney Less commonly they are present in other parts of the tubules and only occasionally in glomeruli The huge cells in various stages of degeneration may fill the lumen of a tubule Desquamation of degenerate inclusion bearing cells is often apparent and readily explains their occurrence in urinary sediment (Fig 2) An interstitial infiltrate of mononuclear cells is almost always present

In the intestinal mucosa, intranuclear inclusions in connective tissue cells and more rarely in epithelial or endothelial cells may be associated with slight superficial ulceration or with severe chronic ulceration The inclusions may also be present in connective tissue cells and vascular endothelium of chronic granulation tissue in other sites, for example in healing burns of the skin

The clinical manifestations of generalized salivary gland virus disease are most clearly defined as they appear in the infant at birth or in the neonatal period The incidence of associated prematurity is high Erythroblastemia and anemia thrombocytopenia, petechiae or ecchymoses of the skin and mucous membranes extreme hepato splenomegaly (often with jaundice) are notable Cerebral involvement as indicated by microcephaly may be present at birth in some cases and the intracerebral calcification most commonly paraventricular, as previously mentioned, may be demonstrated by roentgen rays The manifestations are similar to those of other hemolytic anemias of the newborn infant, especially those caused by maternal isoimmunization (e g Rh incompatibility) Differentiation may depend upon serologic investigations or upon the demonstration of inclusion bearing cells in urinary sediment There is also a close resemblance to congenital toxoplasmosis

The disease in the newborn usually has been fatal within a few days or weeks However, infants have survived in a few instances in which diagnoses had been established by examination of urinary sediment or by isolation of the virus from the urine or hepatic biopsy (MARGILETH,

PUPENKO, 1934) The characteristic cells are shown in a photomicrograph in the publication but they were interpreted as *amoebae* The supposed protozoa were not observed in other organs

In the generalized disease, inclusions may occur in cells of almost any organ in addition to those of the salivary gland Their distribution in the viscera is variable, kidneys, lungs, liver and pancreas (islets of Langerhans) being involved most frequently They are most widespread in the organs of infants dying of the disease in the first days or weeks of life There is usually an interstitial cellular infiltrate in regions where inclusions are found but in some instances this reaction is minimal In the rare cases of the disease in adults the lungs and gastrointestinal tract are the sites of predilection for the inclusion bearing cells (GEILER, 1957)

In the generalized disease present at birth or occurring in the neonatal period, jaundice is frequently present Petechiae and ecchymoses occur in the skin The liver is grossly enlarged and, at times, icteric and there is splenomegaly The cytomegalic inclusion cells in the liver are found almost exclusively in the small bile ducts but may be sparse even when there is a severe hepatitis There is persistent blood formation Foci of necrotic or degenerate hepatic cells and biliary stasis may be present In some instances multinucleated giant hepatic cells are a conspicuous feature There is a marked cellular infiltrate consisting of mononuclear cells and polymorphonuclear leukocytes in periportal regions and to a lesser degree within lobules When the disease in the liver is less severe or apparently of longer duration the infiltrate consists chiefly of mononuclear cells and there may be periportal and intralobular fibrosis

The characteristic large cells with inclusions, when present in the lungs, occur in the epithelium of the alveoli and bronchioles, less frequently in the epithelium of the bronchi and trachea and in the ducts of the mucous glands Focal or diffuse interstitial pneumonia with a mononuclear infiltrate in the alveolar walls is associated frequently with the presence of inclusions However the inclusions may occur in cases in which there is an acute or chronic pneumonia of non specific character An association with interstitial plasma cell pneumonia, attributed to *Pneumocystis carinii*, has been reported in the European literature and reviewed by SEIFERT AND OEHME (1957)

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ROWE AND ASSOCIATES (1956) fortuitously recovered three strains of an apparently identical virus from spontaneously degenerating fibroblastic cells in cultures of human adenoid tissue during studies of the adenoviruses WELLER AND ASSOCIATES (1957) isolated three similar inclusion producing viruses from three infants having hepatosplenomegaly and in two instances periventricular cerebral calcification One of these infants subsequently developed progressive chorioretinitis Cultures of human embryonic skin muscle tissue or of human foreskin were utilized for the isolations

The viruses recovered independently in the three laboratories produce apparently identical cytopathogenic effects in fibroblasts of tissue cultures In original cultures and early subcultures the initial cytopathic changes were small focal lesions often round or oval in shape, consisting of enlarged rounded or ovoid translucent cells contrasting sharply with the uniform sheets of normal fibroblasts The focal lesions appeared in cultures inoculated with suspensions of human tissue or urine after intervals of 5 to 24 days (SMITH 1956 WELLER ET AL, 1957) and in spontaneously degenerating cultures of human adenoid tissue after 22 to 120 days in culture (ROWE ET AL 1956) The foci gradually increased in size and number as long as two months being required for involvement of the entire culture The central cells of the foci became filled with refractile dark granules and then disintegrated leaving masses of granular debris A greenish brown coloration of the granules was noted

In stained preparations of the cultures large intranuclear inclusions are seen in the altered large cells (Fig 3) Some nuclei contain two or three separate inclusion bodies They are finely granular and amphophilic or weakly eosinophilic when stained with hematoxylin and eosin They are separated from the nuclear membrane by a distinct halo and their shape usually corresponds closely to that of the nucleus Small prominent masses of basophilic material are present near the nuclear membrane Occasional cells have two or three nuclei each containing an inclusion In most cells there is a round or oval eosinophilic zone, adjacent to the nucleus and frequently lying in an indentation of the nucleus Infected cells apparently about to undergo dissolution may contain several rather large hyaline amphophilic or eosinophilic bodies

The cellular changes in tissue cultures resemble strikingly those

1955, BIRDSONG ET AL., 1956, WELLER ET AL., 1957) When cerebral involvement was present physical and mental retardation persisted

When the disease appears in infants after the neonatal period or in young children, the clinical manifestations are diverse It occurs most commonly between the second and fourth months of life and with decreasing frequency thereafter In a few instances hepatosplenomegaly, jaundice, anemia, purpura and thrombocytopenia, or microcephaly with intracranial calcification have been noted first when the infant was several weeks or months of age, and it seems highly probable that the infection had been acquired *in utero* With the exception of this rarer, well defined syndrome, the clinical manifestations are variable Clinical pathologic correlations indicate that symptoms may be referable to the varied localization of the viral infection (e g interstitial pneumonitis, enteritis) or to another associated disease Prematurity and a history of nutritional disturbances are common in infants in which autopsies have revealed evidences of generalized cytomegalic inclusion disease

In the less frequent cases in older children and in the rare cases in adults, cytomegalic inclusion disease appears to be usually a superimposed infection during the course of a chronic disease (GEILER, 1957) At present it is not certain whether these cases represent recently acquired infections by the salivary gland virus or activation of latent infections

Propagation of the Salivary Gland Viruses in Tissue Culture

Human salivary gland virus Isolations of the human salivary gland virus in tissue cultures and observations on the behavior of the virus have been reported from three laboratories SMITH (1956), employing roller tube cultures of fibroblasts from explants of human myometrium, isolated two cytopathogenic agents producing intranuclear inclusions The first agent was obtained from the salivary gland of a 7 month old infant Intranuclear inclusions were demonstrated in histologic sections of the salivary glands, but not in sections of other organs A similar agent was recovered from renal tissue of an infant dying with generalized cytomegalic inclusion disease More recently * a third strain has been isolated in the same laboratory from a hepatic biopsy

* SMITH, M G, unpublished

ET AL., 1956). Isolation of strains from tissues or from both tissue and urine of infants ill with a syndrome resembling cytomegalic inclusion disease, and also, from a salivary gland containing intranuclear inclusions, justify further the conclusion that all these agents are strains of the human salivary gland virus.

Each of the investigators has noted evidence of adaptation of the virus *in vitro*. Fluids removed from the original cultures or early serial subcultures contain little or no virus. SMITH (1956) made successful transfers with culture fluid from the original culture only after 30 days of incubation and even then it was necessary to transfer all the fluid, undiluted, from an original culture to each new one. WELLER ET AL., (1957) reported that virus was not demonstrable in fluid removed from original cultures, and both ROWE AND ASSOCIATES (1956) and WELLER AND ASSOCIATES (1957) found it necessary to utilize suspensions of the infected culture cells for early passages of the virus. Considerable amounts of virus are present in the cells before it is released into the culture fluid medium. ROWE ET AL. (1956) demonstrated the delayed appearance of virus in the culture fluid, as compared with the culture cells, in the eighth passage of one strain of the virus. The incubation time for the cytopathogenic effect in test cultures was 44 days when culture fluid from an 11 day old infected culture was inoculated as compared with four days when a cell suspension was inoculated. On the thirteenth day and later, the amount of virus in the fluid, although greatly increased, did not equal that in cell suspensions. After four to ten serial passages, the viruses could be transferred readily with the supernatant fluids from infected cultures in which advance cytopathic changes had developed. Also the incubation period of the cytopathogenic effect decreased with serial passages. In later passages when large inocula of culture fluids were used the length of the incubation periods became much shorter and the initial cytopathic changes were often diffuse instead of focal. However, HARTLEY (1957) has noted the erratic behavior of the human virus even after 35 serial passages, incubation periods and progression to complete involvement of the cultures varying considerably from passage to passage.

Salivary gland virus of mice. SMITH (1954) undertook the propagation of the murine salivary gland virus in explant cultures of mouse embryonic tissue as a model for the subsequent attempts to isolate the human virus. The strain of virus was recovered initially from a pool of three salivary glands from three adult mice of a colony in the Wernse Laboratory of Cancer Research at Washington University

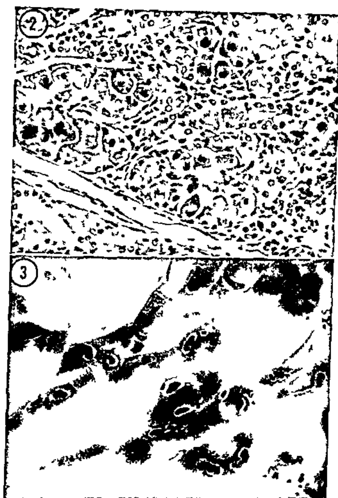


Fig 2 Inclusion bearing cells of renal tubules Desquamated cells with inclusions are free in the lumens Hematoxylin and eosin 230 ×

Fig 3 Cells of tissue culture Enlarged rounded cells from culture of human tissue (explants of myometrium) infected with human salivary gland virus Intranuclear inclusions are present in every cell Bouin fixation Hematoxylin and eosin 400 ×

seen in cytomegalic inclusion disease of infants and in salivary gland disease of animals The similarity or actual identity of strains of the virus isolated by the different investigators was demonstrated by neutralizing and complement fixing reactions with human sera (ROWE

of trypsin dispersed mouse embryonic tissue the character of the focal lesions differed somewhat from those induced by the human virus, in that they first appeared as small holes in the fibroblastic sheet surrounded by the large cells instead of as a compact group of swollen cells

Salivary gland virus of the guinea pig The guinea pig salivary gland virus has been propagated in homologous tissue cultures (HARTLEY, ROWE AND HUEBNER, 1957) The original source of two strains of virus utilized were guinea pigs from two dealers in New York State Each strain was established in serial passages in guinea pigs of a laboratory stock colony Primary inoculations of explant cultures of guinea pig embryonic muscle with infective submaxillary gland suspension (strain I) produced in 10 days small foci of enlarged, round refractile cells in the fibroblastic outgrowth

By the twentieth day, after their appearance in primary cultures, the focal areas had increased in number and extended to involve more than half of the fibroblasts, at 28 days there was almost complete involvement and many cells were necrotic The characteristic changes were reproduced on transfer of fluid containing ground cells to both trypsin dispersed and explant cultures Cytopathogenic changes were not seen on passage from the primary cultures to explant cultures until the twenty fourth day after inoculation but the incubation period in trypsinized cultures was only 6 days and all cells were involved in 12 After 10 passages in trypsinized cultures the incubation periods decreased to two or three days and in later passages, focal or generalized cytopathic changes sometimes occurred within one day In stained preparations made at intervals throughout the passage series, the majority of cells contained large elongated or kidney shaped eosinophilic inclusions within nuclei closely resembling those produced by the human salivary gland virus Definite cytoplasmic inclusions were not observed

Difficulty was encountered in maintaining the second strain of the virus during early passages but after the sixth passage the incubation period became shorter than before and the virus could be readily transferred with fluid containing ground cells Subsequently, in the course of serial passages of two strains of the guinea pig virus in guinea pigs, identical cytopathogenic effects were produced with 10 of 12 suspensions of the salivary glands when inoculated into explant or trypsin dispersed cultures of guinea pig embryonic muscle In these isolations the incubation periods for the cytopathic change varied from three to ten days

Intranuclear inclusions were demonstrated in histologic sections of the salivary gland of each mouse Thereafter the virus was maintained by serial passages at intervals of two or three weeks in young adult mice from a special colony, *free of the spontaneous salivary gland infection* Salivary glands of mice inoculated two weeks previously were used to prepare inocula for tissue cultures Roller tube cultures of embryonic mouse tissue consisting almost entirely of fibroblasts were employed For primary inoculations of the cultures 2 ml of media containing infective mouse salivary gland material (filtrate, Berkefeld N filter) in dilution by weight of 10^{-4} was placed in each culture tube

Many focal cytopathic lesions composed of large rounded refractile cells appeared in the cultures on the sixth or seventh day after inoculation Initially the focal lesions resembled closely those *which have since been produced by the human salivary gland virus* in cultures of human fibroblasts However the foci increased rapidly in size and number By the ninth to twelfth day involvement of the culture was diffuse Infected cells began to degenerate within two or three days after the focal lesions appeared, but a few of the large cells persisted into the fourth week after infection The same type of cytopathic changes developed in subcultures Repeated primary isolations in cultures with infected mouse salivary gland material were successful Even the first passage of the virus to subcultures was readily accomplished with 0.02 ml of culture fluid and was successful also with *0.02 ml of culture fluid in dilution of* 10^{-3}

After three serial passages of the virus in cultures, salivary gland virus disease, as characterized by large intranuclear inclusions and *interstitial mononuclear infiltrate, was reproduced in mice by intra*peritoneal inoculation of 0.02 ml of the culture fluid Fluids used to demonstrate reproduction of the disease in mice represented dilutions of 10^{-11} and 10^{-18} of the original salivary gland material The culture virus could be titrated in the mouse, using the presence of intranuclear inclusions in histologic sections of the salivary gland as an index of infection

HARTLEY (1957) and BRODSKY AND ROWE (1958) readily isolated the mouse salivary gland virus from salivary glands of mice infected with the mouse passage strain of virus used by Smith They employed cultures of trypsin dispersed mouse embryonic tissue In routine passages, employing undiluted culture fluid containing scraped or ground cells, the cytopathic changes occurred in one or two days, and complete involvement of the culture in three or four In cultures

frequently behaved erratically even after 35 serial passages, incubation periods and progression to complete involvement varying considerably from passage to passage

The differences in adaptation of the three viruses are further shown by comparison of amounts of virus in the tissue cells and in the supernatant fluids in the early and later passages The mouse virus multiplies readily and is present both in the culture fluid and the cells in considerable amounts, even in the first passage On the other hand, the guinea pig virus and especially the human virus are released into the culture fluids only in small amounts, if at all, in the early passages, and in later ones the titer of virus in the fluid remains low as compared with that in the cells The behavior of the mouse-virus in cultures may be explained, in part by the fact that the strain of virus, used by all the investigators, has been maintained in mice by serial passages at intervals of two or three weeks over a period of years (SMITH, 1954) Therefore the virulence of this strain may be greater than that of one occurring in the spontaneous disease

The maximum titers obtained by HARTLEY (1957) for the mouse, guinea pig and human viruses in cell suspensions were respectively, $10^{6.5}$, $10^{4.8}$, and $10^{2.5}$ tissue culture infective doses (TCID 50) per 0.1 ml

Specific complement fixing antigen is present in both the supernatant fluids and cell suspensions of adapted cultures of each of the three viruses (ROWE ET AL, 1956, HARTLEY 1957) but of higher titer in the cell suspensions

Range of pathogenicity, cytopathic effect Each of the three viruses induce cytopathic changes in fibroblasts of the homologous species Overt cytopathic effects are not observed in cultures of epithelial cells of various types In explant cultures of homologous tissue containing islands of epithelial growth, intranuclear inclusions, observed in fixed preparations, may occur in those epithelial cells adjacent to affected fibroblasts while most of the others remain unaffected (ROWE ET AL., 1956, WELLER ET AL, 1957, HARTLEY, 1957) In stained preparations of cultures prepared from trypsinized human kidney cells and inoculated with centrifuged infected fluids (human virus), WELLER AND ASSOCIATES (1957) observed isolated, enlarged, rounded cells with intranuclear inclusions, these cells appeared to be of renal epithelial origin

Cytopathic changes are not produced by the human salivary gland virus in cultures of HeLa cells (ROWE ET AL, 1956, WELLER

The virus that had been passed through tissue cultures produced characteristic salivary gland disease in guinea pigs from an uninfected colony and after intracerebral inoculation, acute *meningitis* with intranuclear inclusions in cells of a mononuclear exudate

Comparisons of the Salivary Gland Viruses of Man and Animals in Tissue Cultures

Cytopathic changes The greatly enlarged rounded or oval cells which later become refractile and then disintegrate occur in cultures infected with each of the three salivary gland viruses The focal cytopathic changes induced by the guinea pig and the human viruses in primary and early subcultures are almost identical, a small number of round or ovoid foci composed of a few enlarged cells The lesions extend and increase in number so slowly in early passages that many days or weeks, in the case of the human virus are sometimes required for generalized involvement of the cultures It is only in later passages that focal or, sometimes, generalized changes occur swiftly within one or two days On the other hand, in early passages of the mouse virus the focal lesions are more numerous, less definitely outlined, and progress rapidly to involve the entire culture in two or three days In trypsin dispersed cultures of mouse embryonic tissue the early foci produced by the mouse virus appear as small holes in the culture which result when the characteristic enlarged cells surrounding them pull away from one another (HARTLEY, 1957) *The large intranuclear* inclusions produced by all three viruses have the same characteristics in stained fixed preparations

Adaptation of viruses in cultures Adaptation of each of the viruses to *in vitro* environment is shown by the earlier appearance, more rapid *extension and generalization of the cytopathic changes in* serial sub cultures, and by increasing amounts of virus in supernatant culture fluids Increasing adaptation is more striking for the human and guinea pig viruses than for the mouse virus because it adapts so quickly

HARTLEY (1957) gives the following comparative data Using *undiluted culture fluid* containing scraped or ground cells, the mouse virus after three passages produced cytopathic effects in one or two days, and complete involvement of the cultures in three or four, the guinea pig virus after 10 passages caused changes in two or three days with complete dissemination in 6 to 8 days The human virus

capsules for embedding in the methacrylate to which benzoyl peroxide had been added as a catalyst Polymerization was carried out at 60° C Control cultures were prepared in the same manner

Small portions of the spleens of three week old mice, infected by intraperitoneal inoculation 20 to 48 hours previously, and salivary glands of adult mice, infected by intraperitoneal inoculation two weeks previously, were fixed in a one percent solution of osmic acid in bichromate The tissues were rapidly dehydrated in ethanol solutions and prepared for sectioning in the same manner as were the tissue cultures

Thin sections were cut with glass knives in a Porter Blum microtome, mounted on collodion covered grids and examined in a EMU-2E RCA electron microscope Micrographs were taken at original magnifications of 1000 to 8000 diameters and the prints were enlarged from the negatives as desired

Infected tissue culture cells, human salivary gland virus In electron micrographs some cells thought to be in an early stage of infection were altered from the normal only by an increase in amount of cytoplasm and an unusual globular shape while in others there was a decrease and irregular clumping in nuclear chromatin

Definitely infected cells contained particles which never were observed in uninfected controls In both nucleus and cytoplasm of the enlarged fibroblasts, particles foreign to normal fibroblasts were evident in electron micrographs The nucleoplasm of these cells was almost entirely replaced by a central nuclear inclusion with its peripheral halo In thicker sections of osmic fixed material, examined by phase microscopy and in low power electron micrographs, the nuclear inclusion resembled that seen in hematoxylin and eosin stained sections, a large central dense mass with a surrounding clear zone This appearance was altered in the thinner sections ordinarily examined with the higher powers of the electron microscope here the inclusion appeared as a skein of dense granular material (Fig 4), with small particles interspersed (Fig 5) The amount of osmiophilic material was distinctly reduced in the region surrounding the inclusion When included in the section, the nucleolus was apparently little involved and usually was adjacent to the inner margin of the nuclear membrane In the nucleus the particles thought to represent viral forms were most numerous in the inclusion where they were interspersed among the dense chromatin, but also extended out into the surrounding pale zone These particles varied from 65 to 110 millimicrons in diameter,

ET AL, 1957) No effect is produced in cultures of heterologous tissue or in embryonated eggs by any of the viruses

Physical characteristics of the salivary gland virus of man, the guinea pig and the mouse The salivary gland viruses are also similar in certain physical properties All three viruses show the following characteristics (HARTLEY, 1957) (1) sensitivity to ether, (2) inactivation by heating at 56° C for 10 or 20 minutes, (3) sensitivity to freezing and thawing and prolonged storage at —70° C, (4) filtrability (Selas 03 porosity filter) with some loss in activity, and (5) relative stability from pH9 to pH5 and marked inactivation at pH4 In addition all fail to agglutinate erythrocytes of chickens or homologous species

In spite of the marked similarity of the salivary gland viruses of man mouse and guinea pig in biologic and physical characteristics they appear to be immunologically distinct No antigenic relation has been demonstrated (HARTLEY, 1957) by serum neutralization or complement fixation tests

Electron Microscopy

MINDER (1953) described homogeneous dense particles, measuring approximately 100 millimicrons in diameter and occurring singly or in clumps in the cytoplasm and in the pale zone about the intranuclear inclusion He did not see the particles within the nuclear inclusion, which he described as coarsely honeycombed but not granular Minder made his observations on formalin fixed post mortem pancreatic tissue from an infant having cytomegalic inclusion disease Such preparations are now considered inadequate for electron microscopy because fine structural detail is lost

LUSE AND SMITH (1958) employed electron microscopy for the study of fibroblasts of explant cultures of human myometrium infected by the human salivary gland virus and cells of splenic and salivary gland tissue of mice infected by the mouse salivary gland virus The observations on the character of the intranuclear inclusion and of the particles in the nucleus and in the cytoplasm will be reviewed in some detail

Method Cells of cultures infected 10 days previously were fixed in the roller tubes in Dalton's osmic acid fixative (1% osmic acid in bichromate), dehydrated with graded solutions of ethanol and infiltrated with methacrylate *Finally they were transferred to gelatin*

and comprised a central dense dot of osmiophilic material surrounded by a zone of lighter substance, which in turn, was enclosed by an outer osmiophilic shell of varying thickness (Fig 5) Transitions were seen from these structures to ones in which the central body was so enlarged that it became fused with the outer membrane A less prominent component of the inclusion was an elongated comma shaped particle

Particulate forms in the cytoplasm were numerous and pleomorphic (1) The most conspicuous cytoplasmic particle was a spherical body of dense homogeneous composition 300 to 500 millimicrons in diameter (Fig 6) (2) Smaller target like particles (Fig 6) composed of a central dense structure surrounded first by a clear zone and peripherally by an osmiophilic shell of 20 to [illegible] millimicrons in thickness were present, singly or in nests of many particles These target like particles measured 100 to 180 millimicrons in diameter Both types of cytoplasmic particles were enclosed within vacuolar structures set off from the surrounding cytoplasm by a dense membrane (Fig 6) These vacuoles contained varying numbers of particles sometimes as many as 15 to 30 in the thin section Some large vacuoles or sacs contained both small target like forms and larger spheres The relation between the different forms was not clear

Also large homogeneous dense bodies 2000 to 2800 millimicrons in diameter, were present in the cytoplasm of many cells and small membrane covered blebs 25 to 1500 millimicrons in diameter, were observed at the circumference of these bodies Many of the blebs contained particles, some of which were the dense homogeneous spheres, while others were the target like forms

Dense spherical forms and occasional target like forms were in close apposition to the external surface of the plasma membranes of many cells Usually extracellular particles were not surrounded by a

Fig 6 Electron micrograph of part of cytoplasm of fibroblast from culture of human tissue infected with human salivary gland virus Large homogenous spherical particles (S) surrounded by membranes at the arrows are target like forms with a central dense dot surrounded by a pale zone with a dense shell beyond it They are present as isolated forms or as nests of particles in vacuolar structures 30 000 ×

Fig 7 Electron micrograph of part of cytoplasm of an infected cell of salivary gland of a mouse mouse salivary gland virus There are nests of many target like particles 20,000 ×



Fig 4 Electron micrograph of an infected cell of culture of human tissue infected with human salivary gland virus The nucleus (N) shows central inclusion with skeins of osmiophilic material, surrounded by pale zone (halo of light microscopy) 5000 ×

Fig 5 Electron micrograph of part of intranuclear inclusion (infected cell of human culture) made up of dense granular osmiophilic material and interspersed virus-like particles 42,000 ×.

membrane and probably represented those liberated by the breakdown of infected cells

In electron micrographs of cultures fixed at progressive short intervals (LUSE AND SMITH, in press) after infection particles were first noted *in the nuclear inclusion between two and three days after* inoculation Both nuclear and cytoplasmic inclusions were present in cells of cultures examined 5 to 20 days after infe [illegible]

In cells thought to be in the early stage of inf [illegible] ion prior to the formation of the nuclear inclusion the mitoch [illegible] were increased in number and size as compared with control c [illegible] ture cells In cells in which intranuclear inclusions were fully dev [illegible] and in which cytoplasmic particles were present there were [illegible] fewer mitochondria and sometimes recognizable mitochondria w [illegible] absent from large areas of the cytoplasm

Infected mouse tissue, mouse salivary gland [illegible] *splen cells* The infected splenic cells were mainly reticul [illegible] al The nuclear inclusions, like those of the fibroblasts inf [illegible] ith the human salivary gland virus, were formed by particle [illegible] rspersed with a condensed chromatin skein Particles were [illegible] prominent in the cytoplasm, single dense spheres 250 to 400 m [illegible] crons in diameter contained one or more small round clear foci [illegible] t 60 millimicrons in diameter These structures differed from the [illegible] heres in the fibroblasts infected with the human strain of the virus in that they contained the central clear zones Some of the pale zones contained a single dense dot and thus resembled the target li [illegible] e forms seen in the human culture cells Multiple spheres of variable [illegible] ze similar to those occurring singly were seen in membranous sacs

Salivary gland tissue of the mouse In sections of the salivary glands of older mice inoculated two weeks previously *infected cells with* intranuclear inclusions were readily identified by phase microscopy but they were not numerous In electron micrographs the nuclear inclusion particles were identical with those seen in the mouse spleen and the human culture fibroblasts However, within the cytoplasm of the infected cells there were numerous nests or sacs of the target like particles The sacs of particles measured 2 to 3 2 microns in their greatest diameter and were surrounded by a thin membrane (Fig 7) The enclosed particles measured 120 to 180 millimicrons and appeared identical with the target forms observed in the cytoplasm of fibroblasts infected with the human salivary gland virus

Thus, the nuclear particles associated with both strains of virus


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7

under 6 months of age (especially from the second to fourth month), even when those are excluded in which there was evidence that the infection was acquired *in utero* Thus it would appear that infections in the early months of life are responsible for a disproportionate number of the cases of the generalized disease, and that the age when the infant is first infected is the important factor in determining the course of the disease Other questions arise Is unrecognized intrauterine infection without symptoms in the early neonatal period more common than is suspected at present? Do prematurity, nutritional disturbances, and some infections which have an unexplained association with salivary gland virus infection make the young infant more susceptable to the initial infection as well as to the generalization of the disease?

Spontaneous and Experimental Salivary Gland Virus Disease in Animals

Many older observations and experimental investigations of the salivary gland virus diseases of animals, beginning as early as 1920, are important because they gave information that eventually led to the more recent investigations of this group of viruses

Altered cells characteristic of salivary gland virus infection have been observed in the salivary glands of apparently healthy guinea pigs, rats, mice, hamsters moles, monkeys *(Cebus fatuellus)* and chimpanzees Also JACKSON (1921) noted "organisms" in the ducts of the salivary glands of a dog which were said to resemble those previously described by the same author in the salivary glands of guinea pigs (JACKSON, 1920) Both the intranuclear and cytoplasmic inclusions have been noted in the enlarged cells of the salivary glands in each species, however, the cytoplasmic inclusions are less common in the mouse In the experimental infection in the mouse and guinea pig the recently formed intranuclear inclusions may be smaller than those occurring in infections of longer duration (McCORDOCK AND SMITH, 1936, ROSENBUSCH AND LUCAS, 1939) Also ANDREWS (1930) and ROSENBUSCH AND LUCAS (1939) noted that in the experimental infection the cytoplasmic inclusions appeared in the cell later than the intranuclear one

The location of the altered cells in the salivary glands is not the same in all species In the hamster and mouse they occur chiefly in acini, in the rat with equal frequency in the acini and ducts (KUTTNER

Of the 13 virus positive children, 12 were known to have had complement fixing antibody for at least two months prior to recovery of the virus, and 5 had had antibody 8 to 24 months previously. Thus excretion of virus in the mouth and urine occurred even when antibody had been present for a long time. All of the virus positive children had complement fixing antibody on admission to the nursery and generally the titer remained constant or declined. Virus recovery was not related to the titer of serum complement fixing antibody. Repeated preparations of stained saliva and urinary sediment of these children did not reveal inclusion bearing cells.

As noted by ROWE AND ASSOCIATES (1958), the recovery of virus from the mouth for periods of 2 to 5 months, and as long as 8 to 24 months after antibody was known to be present confirm the chronicity of the infection of the salivary glands in children, as had been indicated previously by the relatively frequent finding of inclusions in the salivary glands at autopsy.

The detection of virus in urine of 7 of the 8 apparently healthy children tested, indicates that dissemination of the virus beyond the salivary glands is relatively frequent in children not showing overt cytomegalic inclusion disease or other obvious clinical symptoms. The presence of virus in the urine of children who had serum neutralizing antibody for periods of 15 to 24 months suggests that the virus may propagate in the kidney during the subclinical infection. This possibility is further supported by the demonstration of prolonged excretion of salivary gland virus in urine of an infant who had survived neonatal cytomegalic inclusion disease (WELLER ET AL., 1957). The long duration of virus excretion in the subclinical infection will make difficult the diagnostic interpretation of virus isolation. The relatively high incidence of inclusions in the kidneys at autopsy when not present in other organs except the salivary glands (SEIFERT AND OEHME, 1957) also suggests that virus may multiply readily in the kidneys.

The occurrence of the infection of the salivary glands as a chronic process with prolonged excretion of the virus in the saliva and urine, together with the prevalence of the disease in young children, provides an explanation for superimposed infections in pertussis, other chronic lung diseases, and debilitating conditions.

The pathogenesis of the disease in young infants is more difficult to understand. A large proportion of the autopsy reports in the literature on generalized salivary gland virus disease are of infants

two or three passages, at best, has always required salivary gland material for the inoculum The species specificity of the salivary gland viruses was also demonstrated in these early experiments

Fatal meningitis with intranuclear inclusions in cells of the exudate was produced in guinea pigs by intracerebral inoculation of salivary gland material (COLE AND KUTTNER, 1926, HUDSON AND MARKHAM, 1932) Non fatal meningitis was produced in the same manner in hamsters, mice and rats (KUTTNER AND WANG, 1934) In only rare instances has brain to brain transmission been accomplished in the guinea pig, and then, only for two or three passages (HUDSON AND MARKHAM, 1932) Intratracheal inoculation of guinea pigs and rats with large doses of infective salivary gland material produced an interstitial pneumonia with intranuclear inclusions (KUTTNER AND T'UNG, 1935) HUDSON AND MARKHAM (1936) demonstrated the greater susceptibility of fetal tissue for the guinea pig virus Intracerebral or intraplacental inoculation of fetal guinea pigs with infected salivary gland material resulted in extensive visceral lesions with intranuclear inclusions ROSENBUSCH AND LUCAS (1939) produced fatal infection in guinea pigs when an unusually virulent strain of the guinea pig virus was injected subcutaneously, into salivary glands, intraperitoneally or into abdominal viscera Necrosis and inclusion formation occurred at the site of infection and inclusions were also observed elsewhere in the body McCORDOCK AND SMITH (1936) produced widespread visceral lesions in young mice by intraperitoneal or intracerebral inoculation of infective mouse salivary gland material Death occurred in four to seven days The most extensive lesions were in the liver, spleen, adrenal and subperitoneal fat and connective tissue Necrosis of tissue and hemorrhage occurred as well as many intranuclear inclusions Less extensive changes were found in the lungs, kidneys, pancreas and intestinal mucosa Although the livers and spleens contained innumerable intranuclear inclusions, emulsions of these organs inoculated intraperitoneally produced no obvious illness, animals autopsied after two weeks showed inclusions in the

[illegible]

glands, might occur in animals just as in man It also seemed apparent that multiplication of the rodent viruses occurred to a greater extent in the salivary glands than in any other site Even when innumerable inclusions were present in the liver and spleen of mice following

AND WANG, 1934), in the mole in terminal tubules near ducts but not in the ducts *per se* (RECTOR AND RECTOR, 1933), and in the chimpanzee most frequently in the acini (VOGEL AND PINKERTON, 1955) In the monkey, *Cebus fatuellus* (COWDRY AND SCOTT, 1935), and in the guinea pig they occur in the ducts

With only a few exceptions, the natural disease observed in animals has been of the latent type Characteristic inclusions were found in the kidneys, as well as the salivary glands in 8 percent of a colony of apparently healthy guinea pigs by MARKHAM (1938) Also KUTTNER AND T'UNG (1935) detected the salivary gland virus in the kidneys of rats and guinea pigs in which inclusions could not be demonstrated Subcutaneous inoculations of emulsions of the kidneys produced inclusions in the salivary glands of young animals of the same species These results indicated that the virus is disseminated beyond the salivary glands and may be excreted in the urine during the subclinical infection in the guinea pig and rat as it is in young children

PAPPENHEIMER AND SLANETZ (1942) reported a spontaneous disease associated with widespread intranuclear inclusions in two guinea pigs of a small colony being used for nutritional studies One of the animals died In their opinion the inclusions were like those occurring in the salivary glands of guinea pigs However, they were unable to transmit the disease to young or adult guinea pigs SMITH AND VELIOS (1950) observed intranuclear inclusions, considered to be of the salivary gland virus type, in many organs of several guinea pigs which had been used in a study of the effects of aminopterin (4-amino pteroyl glutamic acid) One of these animals died The intestinal mucosa was markedly congested and there were many intranuclear inclusions in the epithelium of the intestinal mucosa No attempts were made to transmit the virus

VOGEL AND PINKERTON (1955) observed the disseminated disease in three chimpanzees Inclusions occurred in the salivary gland, adrenal cortex and, in one instance, the myocardium Two of the animals were ill, one with diarrhoea of two weeks duration, the other with persistent diarrhoea for two months and frequent upper respiratory infection

Serial transmission of the virus with production of inclusions in the salivary glands in animals of the homologous species was demonstrated for the guinea pig (KUTTNER, 1927), and the rat, hamster and mouse (KUTTNER AND WANG, 1934, KUTTNER AND T'UNG, 1935) In the experience of all investigators, serial transmission for more than

glands in titers of 10^2 to 10^5 No definite correlation was obtained between viral titer and number of inclusions observed

The chronic subclinical infection in mice is comparable to that in young children in the persistence of virus in the salivary glands and excretion of virus in the saliva over long periods Failure to recover virus from urine of mice after the acute phase of the infection is in contrast to the frequency of recovery of the human salivary gland virus from the urine of children over periods of months The finding of inclusions in the kidneys in addition to the salivary glands in 8 percent of apparently healthy guinea pigs (MARKHAM, 1938) suggests that the behavior of the guinea pig virus may be more analogous to the human virus in this respect, than is that of the mouse

Prevelance of antibody in several stocks of guinea pigs While developing the complement fixation test for the guinea pig salivary gland virus, HARTLEY (1957) found that all the available commercial complement contained complement fixing antibody for the guinea pig virus in titers of 1 64 or above Therefore, serum from young guinea pigs of a stock apparently free of infection was used as complement in all tests involving the guinea pig virus The prevalence of antibody in several stocks of guinea pigs was studied and correlated with the presence of virus as indicated by inclusion body formation (HARTLEY, 1957)

In a stock of guinea pigs with relatively low incidence of infection no inclusions were found in animals under 5 months of age Before this age there was a low incidence of complement fixing antibody of low titer (1 8) and the frequency of antibody was lower in animals between the age of four weeks and five months than in animals three weeks of age Therefore, this antibody of low titer was interpreted as residual maternal antibody

The majority of infections detectible by serologic or histologic methods apparently took place after the fifth month of age, since there was a significantly higher incidence of complement fixing antibody in guinea pigs 8 months of age and older than in the younger animals However inclusion bodies were only rarely noted

The age distribution of inclusions and antibody was also determined in a colony with a higher incidence of natural infection In this colony detectible infection tended to occur earlier in life than in the colony with a low incidence Inclusions were not present in animals less than four weeks of age Complement fixing antibody in low titer, presumably residual maternal antibody, was present in 58 per-

intraperitoneal injection of the mouse virus, serial transmission with suspensions of these organs could not be accomplished

HARTLEY (1957) made a quantitative study of the multiplication of the mouse virus in the liver and spleen following intraperitoneal injection of the virus She demonstrated that a degree of multiplication occurred in these organs but the titer of virus, determined in tissue cultures, did not reach that required to produce a fatal infection in a subsequent passage The extent of multiplication was dependent on the titer of the original inoculum, the highest titers being detected in the livers and spleens of mice succumbing to acute disease following intraperitoneal inoculation of large doses of virus When a sublethal dose of virus was injected intraperitoneally, virus was detected in the liver and spleen throughout a period of 10 days but did not attain a titer necessary to produce a fatal infection

Although a degree of multiplication of the virus in hepatic and splenic cells has been demonstrated, the reason for the difference in the behavior of the virus in these cells as compared with that in the salivary glands is not yet clear

Experimental chronic subclinical infection in mice BRODSKY AND ROWE (1958) established a subclinical infection with the mouse salivary gland virus in three week old mice from a colony free of the spontaneous infection Their purpose was to study the chronic infection in mice as an experimental model for the human salivary gland virus infection The infected mice were maintained for a year and at intervals during this time, two to four mice were removed for study Mouth swabs, urine and submaxillary glands were obtained from individual mice, and virus isolations on tissue cultures were attempted from these materials Histologic sections of salivary glands were examined for intranuclear inclusions

The first isolation of the mouse salivary gland virus was on the eighth day after inoculation and coincided with the appearance of virus in oral swabs and of inclusions in the submaxillary gland Maximum titers of virus were obtained from the salivary glands on the twelfth day, only at this time was virus recovered from the urine After one year the submaxillary glands of three mice were still positive for salivary gland virus and the two of the mice tested were excreting virus in the saliva

Inclusion bodies were most numerous during early stages of infection They were present in mice examined at 60 days but not at 120 days or thereafter although virus continued to be present in the

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cent of the sera of these young animals In animals between the age of four weeks and 8 months the frequency of inclusions paralleled that of antibody, reaching an incidence of 33 percent After one year of age the incidence of inclusions decreased but that of complement-fixing antibody increased to 92 percent

Detection of virus in the salivary glands or saliva of guinea pigs has not yet been correlated with the presence or absence of inclusion bodies

The remarkable similarity between the subclinical infections in man and animals demonstrated by recently available laboratory techniques should make it possible to use the animal infections for experimental studies related to the pathogenesis of the generalized disease in man Moreover the salivary gland viruses provide excellent models for study of viral infection of cells Their advantages in this regard include their absolute species specificity, the distinctive and readily recognized morphologic changes which they induce in the cell and the gradual progression of these cellular changes in tissue cultures All the above features make these viruses worthy objects of continued study

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Viruses of the Respiratory Tract

By Thomas G Ward

Introduction

An interesting phenomenon has occurred among the respiratory virologists during the past five years The utilization of new tissue culture technics has demonstrated a Pandora's box of agents which has left this group of virologists reeling under a series of new abbreviations and has precipitated conference (1, 20, 21, 22) after conference in an attempt to crystalize the knowledge available This review is one of several reviews (2) and is an atempt to clarify and possibly summarize some of the information which has been gained in such a short time

The search for the virus of the common cold is no longer considered sophisticated, rather the erudite virologist now hopes that he can keep the number of viruses which cause symptoms of the common cold to a manageable size Certainly there are at least a few dozen The change in emphasis has occurred in many laboratories and more people are working on the causes of the common cold than were interested in such endeavors five to ten years ago The symposium "Viruses in Search of Diseases" (1) is a classic example which has resulted from the confusion One can list the adenoviruses,

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virus grows in a susceptible cell in the anterior nares, multiplies and proceeds posterior into the nose involving more and more cells until a sufficient number have been involved that clinical symptoms may ensue The swelling of the nasal mucosa reported by many

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and the ocular systems are chiefly affected and the 6 diseases include acute respiratory disease (ARD) of military recruits, follicular conjunctivitis, keratoconjunctivitis, nonbacterial pharyngitis in children, pharyngoconjunctival fever, and viral pneumonitis (atypical pneumonia without cold agglutinins)

of diseases In discussing this point, one needs to establish a theory of pathogenesis of the mild respiratory group of diseases

Certainly the pathogenesis of influenza and the pathogenesis of pharyngoconjunctival fever are different as the production of disease caused by adenoviruses 1 and 2 in small children—a mild type of upper respiratory infection—is infection of the nasal mucosa or conjunctiva followed by generalized disease

Laboratory experimental work (4) with the influenza virus has indicated that the disease begins in the lower respiratory tract with involvement of the bronchiolar epithelium As the virus is released from the cell, it is swept upward by the action of the cilia involving more and more cells until the striated columnar epithelium overlying the cartilagenous bronchi are reached These cells are infected and give rise to large numbers of virus particles with the result of systemic disease manifested after 2 to 5 days incubation period

If one streaks the conjunctiva of a volunteer with adenovirus type 4 on the left side, interesting phenomena occur The left side of the volunteer's nose becomes reddened and inflammed with swelling of the nasal mucosa, followed by involvement of the left tonsil and the left side of the throat Thus is a distinct clinical phenomenon which one can observe for as long as 24 to 36 hours, after that the right side of the nose becomes involved and also the right tonsil and the right portion of the throat This has been referred to as "unilateral experimental respiratory disease" In this instance, of course, the cells involved, are the cells which are in immediate contact with the virus, the lining cells of the conjunctival sac, the lining of cells of the nasal mucosa and those overlying the tonsilar and pharyngeal areas Drainage through the lymph system involves the satelite node anterior to the auricular appendage

Patients often complain of a common cold beginning in the anterior portion of the nasal cavity and progressing down the respiratory tree leading to a cough and finally involvement of the vocal cords and then the chest area resulting in more systemic disease The two groups of agents noted above are different from one another in clinical manifestations The localizing symptoms are the running nose and choryza which preceed the systemic symptoms in the adenovirus infection whereas in influenza these symptoms often follow the systemic disease

Is it not reasonable to theorize that the pathogenesis of many of the agents causing mild respiratory disease is about as follows? The

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these agents were breaks of conjunctival fever out and an effect), Western Europe (31) and in field epidemiol he original outbreaks (33, 34) new group of vir reas the outbreak observed in indicates that the) There have been sporadic indled on a large wherein types 1, 2, 5 and 6
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fections of the ade e epidemics of this particular demonstration th 7, 28, 30, 31, 37-40), whereas and atypical pneumo ter (11 33, 41) but is usually about extensive stud epidemics in the summer time causes of disease in m lakes or swimming in pools, after confusion resulted unities
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Patients often complain of a common cold begin anterior portion of the nasal cavity and progressing dowr tory tree leading to a cough and finally involvement cords and then the chest area resulting in more systemic two groups of agents noted above are different from or clinical manifestations The localizing symptoms are the r and choryza which preceed the systemic symptoms in th infection whereas in influenza these symptoms ofter systemic disease

Is it not reasonable to theorize that the pathogenes the agents causing mild respiratory disease is about as follow

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It is of interest that these same type symptoms were produced in the early volunteer studies (6) by inoculation of the conjunctiva Several illnesses reported in the past were very likely pharyngoconjunctival fever One such outbreak (26) occurred in 1943 and was referred to by the authors as "Swimming Bath Conjunctivitis" and another outbreak (27, 28) in Colorado in 1951 which was called "Greeley Disease" also represented adenovirus infections

After 1955 there were many outbreaks of conjunctival fever observed in Canada (29), England (30), Western Europe (31) and Japan (32) and it is of interest that of the original outbreaks (33, 34) all were due to type 3 adenovirus whereas the outbreak observed in Canada was due to types 7 and 14 (35) There have been sporadic cases of pharyngoconjunctival fever wherein types 1, 2, 5 and 6 (23, 29, 36) have been isolated

The time of occurrence of the large epidemics of this particular disease have been in the summer time (17, 28, 30, 31, 37–40), whereas the ess may be prevalent in the winter (11, 33, 41) but is usually idemic In many instances the epidemics in the summer time n due to either swimming in lakes or swimming in pools, rly those maintained by communities

Acute Respiratory Disease (ARD)

6, the Commission on Acute Respiratory Diseases of the ces Epidemiologic Board in order to attempt to clarify the ith respect to the many mild respiratory diseases offered f classification which was adopted at that time The term ory Disease was suggested for "a specific disease single or to a group of closely related agents", and d the common cold, the afebrile common cold and n cold along with primary atypical pneumonia as a ute respiratory diseases

t (16) of the adenovirus group of papers indicated ry disease was caused by type 4 adenovirus in

of diseases In discussing this point, one needs to establish a theory of pathogenesis of the mild respiratory group of diseases

Certainly the pathogenesis of influenza and the pathogenesis of pharyngoconjunctival fever are different as the production of disease caused by adenoviruses 1 and 2 in small children—a mild type of upper respiratory infection—is infection of the nasal mucosa or conjunctiva followed by generalized disease

Laboratory experimental work (4) with the influenza virus has indicated that the disease begins in the lower r involvement of the bronchiolar epithelium As from the cell, it is swept upward by the action o more and more cells until the striated columnar e the cartilagenous bronchi are reached These ce give rise to large numbers of virus particles with t disease manifested after 2 to 5 days incubation pe

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virus grows in a susceptible cell in the anterior nares, multiplies and proceeds posterior into the nose involving more and more cells until a sufficient number have been involved that clinical symptoms may ensue The swelling of the nasal mucosa reported by many investigators, and the watery discharge early in most mild respiratory diseases indicate that the cells involved are largely in the nasal mucosa After the passage of a few hours, the throat symptoms begin to appear and the clinical manifestations of that area are apparent, after another few hours the larynx may become involved and then the lower portion of the respiratory tract

With as many as 20 to 30 different viruses capable of causing symptoms of the common cold, can one be hopeful of producing an effective vaccine? Are there too many agents? Can the reticuloendothelial system handle this number of antigens in a single inoculation? Would the investigator find himself in the position of having protected against several agents only to have one that he had not protected against come in to infect the susceptible cells, which were then available for infection by the agent against which he had not protected In more simple terms, could one protect against 8 or 10 different viruses and have an 11th virus come in and bring about disease which 11th virus would not have brought about the disease had the individual not been vaccinated because one of the previous ten would have caused the same type of clinical illness? Will there be antigenic overing to such an extent that a vaccine may be effective however? answers to these questions may be secured in vaccine attempts, volunteers but in epidemiological field trials also

The Adenoviruses

e a large group of agents which have been delar, the respiratory and the intestinal systems of e higher primates, particularly the chimpanzee ypes are known to affect man and there are at ses produced by these agents The respiratory s are chiefly affected and the 6 diseases include ase (ARD) of military recruits, follicular con nctivitis, nonbacterial pharyngitis in children, fever, and viral pneumonitis (atypical pneuglutinins)

In experimental disease produced in volunteers (6), these agents characteristically produce an inflammation of the conjunctiva and the submucosa of the nose, there is fever, accompanied by follicular hyperplasia of lymphoid tissues and enlargement of regional lymph nodes (7) In infant pneumonitis, occasional fatalities have been reported (8 10), but these are quite rare On occasions severe epidemic keratoconjunctivitis may be followed by permanent damage to the cornea It is important to emphasize that these are the exceptions, however, and that adenovirus diseases are rarely fatal (5) and are self limited

The adenoviruses and the diseases which they produce give the respiratory virologist a justifiable sense of accomplishment In 3 years these agents were isolated, the criteria outlined above were carried out and an effective vaccine was demonstrated in volunteers (12) and in field epidemiologic trials in military recruits (13, 14) No other new group of virus agents were so characterized so rapidly This indicates that the new tissue culture applications are effective when handled on a large enough scale to be used properly

The first reports (15) indicated that these agents were masked infections of the adenoids and tonsils, this was followed shortly by the demonstration that they were the etiologic agents for ARD (16) and atypical pneumonia in military recruits These reports brought about extensive studies of the attributes of these new agents as the causes of disease in man The term adenovirus (17) was agreed upon after confusion resulted with the use of several terms (RI, ARD, and APC) Other agents (18, 19) were reported independently by other workers but these have now been shown to be adenoviruses

Adenovirus Diseases

Pharyngoconjunctival Fever

This disease has 3 major clinical manifestations—fever, conjunctivitis and pharyngitis, and these 3 manifestations were first observed in a laboratory infection (5) wherein type 3 adenovirus was recovered from the throats and conjunctiva of accidentally infected laboratory workers Because of the newness of the adenoviruses at this time, it was decided to investigate other outbreaks of pharyngitis and conjunctivitis, characterized by malaise and preauricular lymph node involvement The first outbreak of these was described (24) in

1954 among children and adults who became ill in the clinical center at the National Institutes of Health in Maryland The authors described the illness as being characterized by pharyngitis, rhinitis, conjunctivitis, by cervical lymphodenopathy and by temperatures which ranged as high as 104°

It is of interest that these same type symptoms were produced in the early volunteer studies (6) by inoculation of the conjunctiva Several illnesses reported in the past were very likely pharyngoconjunctival fever One such outbreak (26) occurred in 1943 and was referred to by the authors as 'Swimming Bath Conjunctivitis" and another outbreak (27, 28) in Colorado in 1951 which was called "Greeley Disease" also represented adenovirus infections

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The time of occurrence of the large epidemics of this particular disease have been in the summer time (17, 28, 30, 31, 37–40), whereas the illness may be prevalent in the winter (11 33, 41) but is usually not an epidemic In many instances the epidemics in the summer time have been due to either swimming in lakes or swimming in pools, particularly those maintained by communities

Acute Respiratory Disease (ARD)

In 1946, the Commission on Acute Respiratory Diseases of the Armed Forces Epidemiologic Board in order to attempt to clarify the situation with respect to the many mild respiratory diseases offered (42) a type of classification which was adopted at that time The term Acute Respiratory Disease was suggested for "a specific disease process due to a single or to a group of closely related agents", and they also suggested the common cold, the afebrile common cold and the febrile common cold along with primary atypical pneumonia as a classification for acute respiratory diseases

An early report (16) of the adenovirus group of papers indicated that acute disease was caused by type 4 adenovirus in

military recruits Subsequently, it was shown (17, 27, 43-46) that types 3 and 7 were also implicated in producing a large number of the acute febrile respiratory diseases suffered by the military recruits of the Army, Navy and Air Force in the United States Subsequently, the observations were extended to the British (35) and to the Royal Netherlands Army (20, 25) where another type, type 14, was incriminated as the cause of this disease in young military personnel

It is of interest that the term, namely acute respiratory disease, refers to catarrhal fever of the United States Navy ("cat fever") and to "febrile catarrh" as described (47) by the English workers

Some of the early studies (48) indicated that approximately 90% of all of the acute respiratory diseases occurring among young military personnel could be attributed to the adenoviruses However, later studies (17, 27, 49) by other workers demonstrated that a large portion of these diseases could not be accounted for by this group of agents As a matter of fact only about half of the cases were reported as caused by adenoviruses It seems reasonable that a sizeable proportion of acute respiratory diseases in military recruits are of unknown etiology and will await the discovery of more agents or of many of the agents which are now in the deepfreezes of virologists to be classified and characterized to determine their role in etiology of these diseases

The clinical features of acute respiratory disease in military recruits vary from the symptoms (50) of the ordinary common cold to a rather severe generalized respiratory disease which requires hospitalization The most common and most frequent symptom and sign is referable to the upper respiratory tract namely, pharyngitis and rhinitis Occasionally one sees conjunctivitis and otitis media may be a complicating factor In the more severe cases there may be rather extensive involvement of the throat with extension downward into the respiratory tree leading to the production of tracheobronchitis In one rather interesting study (27) it was suggested that there should be 5 categories of disease, these categories being based on the anatomic location of the major signs and symptoms The authors suggested that these be labeled respectively, febrile common cold, non streptococcal pharyngitis, tonsillitis, bronchitis and primary atypical pneumonia It is of interest that the group of diseases which yielded the largest percentage of adenoviruses in this p [illegible] from those which were diagnosed as fel [illegible] centage yielding adenovirus was 48, wh

agents was isolated from 38% of cases of tonsillitis As stated above, this would mean that a large proportion of the number of cases of acute respiratory disease are still of unknown etiology or at least our technics for isolating the adenoviruses are not sensitive enough in this instance or the laboratory procedures need to be utilized more extensively

Adenoviruses, types 4 and 7, have not been seen (27, 51) in civilian populations or in civilian outbreaks This has posed a dilemna for the epidemiologists but it must be apparent that the situation for spread of the virus is different in the military population Reports (5, 52) have demonstrated approximately 20 to 50% of the persons entering military training to have antibodies against types 4 and 7 and in another study the inmates of a state reformatory of essentially the same age group was shown to have similar antibodies

It is of particular note that fairly extensive studies (52, 53) among college student populations in the United States have shown an extreme paucity of the diseases due to adenoviruses—the order of 2 to 8%. There have been reports (54) from the Netherlands to the effect that a disease characterized by symptoms not unlike those of influenza was observed in industrial groups in that country, however, while only 20% of the cases were shown to be caused by influenza virus, 80% had a significant serologic rise to the antigen shared by the adenovirus group The study did not state the specific type of adenovirus involved in the outbreak

The acute respiratory disease problem in military recruits could be rendered essentially at an end if the vaccine, which will be discussed below, was employed by all of the military populations The problem of acute respiratory disease in civilians however, is still of paramount importance and the etiologic agents are unknown in large measure

Epidemic Keratoconjunctivitis (EKC)

Recently a symposium (20) on viral keratoconjunctivitis reviewed fairly extensively the adenovirus infections of the eye and will not be repeated in this report, but rather the summarizing comments will be included herein In 1955 type 8 adenovirus was demonstrated (55) in a case of epidemic keratoconjunctivitis which was classic in nature and could hardly be doubted as a valid case of this disease There was a curious question as to whether a single case of this type could have any particular significance, however, subsequent

studies (56) quickly established that classical EKC in Switzerland, Germany, Italy, Austria, Japan, Canada and the United States were caused by adenovirus, type 8 and the criteria indicated above could be carried out by the reproduction of the typical illness (57) in volunteers with this agent Other eye-diseases particularly those of a conjunctivitis nature have been reported as caused by this group of agents Surveys on patients (29, 58) with follicular conjunctivitis seen in Ophthalmologic Clinics demonstrated that 32 strains (29) of adenoviruses, namely types 2, 3, 7 and 9 were able to be isolated from eyewashings of 55 patients with this disease, whereas another author (18) reported 13 isolations of types 2, 3 and 6 from 27 patients with conjunctivitis of a follicular nature in the United States Children in Saudi Arabia who had acute conjunctivitis yielded (59) 12 strains of adenoviruses, particularly types 3, 7 and 8 This study is of interest because 3 new types of adenoviruses were also demonstrated in the eyes of these children and they have now been designated as types 15, 16 and 17

One author (60) has attempted to distinguish the corneal lesions which have been observed in the cases of follicular conjunctivitis from those of classical EKC on the following 3 points 1 that the lesions of EKC are subepithelial while those of pharyngoconjunctival fever are epithelial primarily, 2 the corneal changes which occur in pharyngoconjunctival fever appear at the same time as the conjunctival changes while the conjunctival changes in EKC may be delayed as long as a week, and 3 there may be more permanent corneal damage in the EKC infections than in the ordinary follicular conjunctivitis

Acute Febrile Pharyngitis

This particular disease was shown in early studies (5, 51, 61) to be caused by type 3 adenovirus and on the basis of several investigations by many authors, types 1, 2 and 5 appear as the adenovirus infections most likely to produce this particular group of diseases In a family outbreak (62) in the United States, the type 2 virus was indicated as the etiologic agent and other workers (63, 64) were able to produce acute febrile pharyngitis in volunteers using types 1 and 5 Other investigators (36, 51, 65) have demonstrated that types 1, 2 and 5 may produce a type of nonbacterial pharyngitis In the well planned epidemiologic analyses (11) of an orphanage nursery near

Washington, D C, acute febrile illnesses in 40 children with type 1 infections, in type 2 infections of 33 individuals and 21 with type 5 infections, indicated that there was a close association between the isolation of the agents and the febrile illness which was demonstrated in the child concurrant with his adenovirus infection In this particular study it was of interest that if children had serum neutralizing antibodies against types 1, 2, 3 and 5 these individuals did not have infection attack rates as high as the children who happened to enter the orphanage with less antibodies against these types The epidemiologic analyses indicated that if a child had no antibodies against type 2 but did have antibodies against type 1 3 and 5, he was less likely to have disease against the former than if a child had no antibodies against the latter group Prior infections with types 1, 2 and 5, however, did not appear to influence the attack rate of type 3 infections while the children were in the orphanage, but if they had antibodies against types 2, 3 and 5 it seemed as if there was a reduced risk against having infection with type 1 adenovirus

It is of interest that types 1 and 2 represent apparently the earliest infections (5) which infants and small children get with adenovirus In this age group they may produce a variety of symptoms ranging from the afebrile common cold with rhinorrhea to a severe pneumonitis which might actually result in death Serologic surveys (51) carried out early in the adenovirus studies indicated approximately 80% of the children under one year of age in crowded urban areas showed evidence of infection by the time they were 5 years of age and that in a less crowded suburban population this was reduced only to 40 or 60% particularly by the time the children reached the age of 10 It seems that types 3 and 5 infection occur at a later age than types 1 and 2 and that types 4 and 7 are rarely seen until the individuals reach late teen age On the basis of these data it would appear reasonable to assume that the most important medical problem with respect to adenoviruses for the civilian practitioner lies in the area of pediatric medicine A vaccine containing types 3 4 and 7 is now being rather extensively used by pediatricians in America

Studies of Adenoviruses in Volunteers

During the months just following the isolation of these agents, several hundred volunteers (6, 12, 57, 63, 64) were utilized in assisting

the investigators in demonstrating that pharyngoconjunctival fever, acute febrile pharyngitis follicular conjunctivitis and epidemic *keratoconjunctivitis* were *distinct* diseases and could be dilineated with the volunteer under experimental conditions

Further the volunteers were utilized in testing experimental *vaccines* (12) *and then demonstrating that* the level of antibody demonstrable in the circulating serum was inversely related to the production of disease in these individuals

One of the primary problems in volunteers has to do with the production of objective disease which may be seen by any observer and even by the volunteers themselves One of the most important advances made *in the group of adenoviruses infections has to do* with the fact that the investigators were able to demonstrate (6) that conjunctival inoculation, that is by swabbing the lower conjunctiva, resulted *in a distinct and interesting conjunctivitis after 3 or 4 days* and that this conjunctivitis was an objective disease that could be demonstrated to one's satisfaction Studies of a coded nature where *in the clinical observers were not aware of the type of inoculum given* to the volunteers resulted in data of a high order of magnitude, when this type of inoculum procedure was employed

Adenovirus Vaccines

The first time that adenovirus vaccine was shown to be effective was in a volunteer experiment (12) utilizing formalin inactivated *type 3 virus and this study* was subsequently followed by successful field trials of similar prepared vaccines in military recruits The vaccines, grown on monkey kidney, are formalized and filtered in a similar manner to the methods employed in the production of polio myelitis vaccine The subsequent studies (13) showed that an experi mental vaccine containing types 4 and 7 was efficacious, particularly in preventing ARD in military recruits In one study (13) it is of interest that significantly few of the respiratory illnesses occurred in the vaccinated group, namely 4 8% ,whereas those in the un vaccinated group were hospitilized at the rate of 23 8%

Types 3, 4 and 7 were successfully employed (14) in a trivalent vaccine amongst Naval recruits at the Great Lakes Naval Training Center in Illinois and not only was there a significant protection against ARD but there also was a significant protection against pneumonias

due to type 4 adenovirus It is of interest that type 4 was the only adenovirus isolated in that particular study in April 1956 There was a significant increase in the serum neutralizing antibodies produced against all three types in the vaccinated individuals

Vaccines containing types 1, 2, 3 and 5 are being tested (11) in infant nursery groups and it is of interest that these studies can be done utilizing a vaccine containing 4 different viruses

Acute respiratory disease can be prevented in military recruits if adenovirus vaccine is employed early in their admission to military service The successful isolation, characterization and demonstration that a successful vaccine may be produced by the adenoviruses in a little less than 3 years augurs well for this type of approach in experimental medicine

Properties of Adenoviruses

Since the discovery of adenoviruses approximately 18 different types have been recovered in human beings, 4 types have now been demonstrated from monkeys and one has been shown in chimpanzees (66) It seems that these agents have a difference in tissue culture host range and it has been recommended by some workers (66) that adenoviruses of monkey origin should be preceeded by the prefix "M" and those of the chimpanzees should be preceeded by the prefix "C" It is of interest that other groups of agents known as the cytopathogenic group No 1 which have some attributes of adenoviruses, have not been classified as yet (67) as to whether they might or might not be adenoviruses

The adenoviruses are a rather large group of agents which share a group specific complement fixing antigen, they are cytopathogenic for tissue culture and they do not produce disease so far as can be ascertained in the small laboratory mammals and further they are ether resistant In addition to the 18 strains noted above, evidence (66) has recently been reported by several groups of workers to indicate that there may be reciprocal neutralization procedures with rabbit antisera indicating type 7 strains to be not distinct but there are some others in type 7 which have been referred to as ' Goman", and this subtype has been referred to as type 7a

Some evidence has indicated that there may be subgroups of the adenoviruses which are biologically related These data are based on the type of cytopathogenic changes which may be seen in HeLa cell

cultures along with the cytologic ones that are also seen in monkey kidney culture The quantitative aspect of neutralization procedures and their epidemiologic behavior indicate that types 1, 2 and 5 may be quite closely related and that types 3, 4 and 7 are also in a similar category Other workers have suggested however that further study is needed before one should attempt any formal subgrouping of this group of agents

Stability

Adenoviruses may be stored at about 4° C for long periods of time (61, 68) and as they can accept the pH range from 2 to 10 they are very stable with respect to H ion concentration The destruction (69) of adenovirus type 3 is accomplished by great rapidity by chlorine and the inactivation rates are essentially those seen for *Salmonella typhosa* They are also inactivated (12, 13) by formulin in concentrations from 1 400 to 1 4000 and in this instance the antigenicity is not affected It has been demonstrated however relatively unstable (30, [illegible]

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Size

By a technic of electron microphotographing sections of infected HeLa cells, workers (71–73) were able to indicate that the adenovirus particles had a diameter of the order of 50 to 65 millimicrons, whereas other workers (70) utilizing a variety of different kinds of procedures, such as gradacol membrane filtrations, electron microscopy of the uranium shadowed purified virus and pseudovolocity sedimentation technics, indicated estimates of size range from 80 to 120 millimicrons in size Most of these studies of the latter group were done on either liquid virus or on the viruses extracted from cells and thus did not seem to make much difference in the estimate of sizes Other workers (76) concentrated adenovirus preparations and then studied them by electromicroscopy and in addition to the 60 millimicrons particles, they also reported several particles of a larger diameter and in addition observed particles which had long, slender filaments and also noted particles of a 5 sided nature It is of interest in this particular study that the virus preparation was prepared from infected monkey kidney and one of the possible criticisms of this study may be that

there might have been contaminated virus of monkey origin or one of the simian agents may have been present, yielding different kinds of particles which were seen in the preparation

Host Range

The host range of the adenovirus has been most extensively studied using tissue culture technics All of the known adenoviruses, at the present time, will grow in the HeLa cell and also in monkey kidney There is a production of cytopathogenic changes in these 2 tissues and propogation proceeds at an acceptable rate It is of interest that the adenoviruses of human origin without special attempt cannot be propagated for long periods of time in serial passage in monkey kidney cells, but the first 6 types were adapted (74) by means of using a concentrated inocula Some of the higher types, namely 10, 12, 13, 15, 17 and 18 adapt rather poorly (39) to growth in tissue cultures and also require concentrated heavy inocula before they could be established A rather extensive study (15, 61) of type 2 adenovirus indicated that this particular agent was able to be grown in a number of human and animal tissue cultures, including those from hamsters, chick embryoes, cotton rats rabbits and human embryoes This same study indicated that types 3 and 4 are somewhat less ubiquitous in their host range and as demonstrated by the fact that types 3 and 4 would not propogate on rabbit trachea (61) and cotton rat tissue cultures whereas types 1 and 2 were able to be propogated on these particular cells One of the interesting aspects of host range has to do with the fact that adenoviruses may produce a large and rather marked cytopathogenic effect without the production of extensive amounts of infectious virus This is particularly noted with type 5 in rabbit fibroblast, (75) with types 3, 4 and 7 (77) in bovine tissue cultures and types 3 and 4 in rabbit trachea (61)

A rather interesting observation (75) was made to the effect that type 5 could be recovered from a rabbit spleen which had been removed as late as 55 days after intravenous inoculation of the rabbit It was of interest in this experiment further that the virus could be demonstrated only by growing the spleen in tissue culture and that tissue suspensions into HeLa cell cultures yielded negative results These data are quite similar to the data observed in human adenoids early in the isolation when one could demonstrate a virus present in the adenoid by tissue

culture technics by growing the tissues and allowing them to degenerate, but if one took the same adenoid (15) and ground it up and inoculated a susceptible cell, there was no demonstration of the virus

While it may be stated that there are no small laboratory mammals which are capable of being infected with adenoviruses, the statement should be that the viruses have been found to be really nonpathogenic A wide variety of animals by several routes have been inoculated (16, 61) without the production of disease but there is a suggestion because of antibody responses after a single inoculation such as seen in guinea pigs, cotton rats and in hamsters, that there very well may be an infection produced (78) There have been some guinea pigs (65) that have been shown to have naturally occurring complement fixing antibodies to adenovirus which indicates that they must have come in contact with the antigen at some time

The Interaction Between Adenoviruses and Cells and the Quantitation of Adenoviruses

Many workers have indicated discrepancies from different laboratories on the ability of the HeLa cell to be infected by adenoviruses and these difficulties in comparison with results from different laboratories and in the interpretations of the data may have at least 4 distinct reasons for such discrepancies Probably the most important of these reasons has to do with the methods of titrating the viruses For instance, it is seen that there is a linear relationship (65, 79, 80) when one titrates in HeLa cells between dilution of the virus inoculated and the time of appearance of the sited pathogenic change It has been demonstrated by several groups of workers that a ten fold dilution of the virus results in a prolongation of the incubation period of the order of 1 2 to 2 days Sometimes dilutions of a limiting nature may not produce effects (39) for as long as 20 to 30 days and then it may be positive only on the next passage

The second reason for these discrepancies lies in the fact that HeLa cells differ in their sensitivities and in their abilities to detect the viruses This has been demonstrated by groups of workers (57) and it is obvious that titration procedures must not only utilize essentially the same periods of observations, but they must also utilize the same kinds of cells It has been demonstrated that short periods

of observation give only a slight indication of the amount of virus present and do not show the total situation at all

The third reason probably lies in the difference (84) between titers produced by different adenovirus types in different laboratories, and here one should be extraordinarily careful to discuss the relative titers of the virus to the actual titers involved The short observation titrations are excellent for comparison of different amounts of virus of the same adenovirus type since the observations are stopped during the period when the growth curve is still logarithmic But one cannot accept short observation times, when one is trying to compare adenoviruses of different types, because to do so is only to be misleading unless one actually studies the relative titer to the actual titer In this connection, it is important to keep in mind that one should use titrations (39, 82, 83) above the order of 1 to 100 because sometimes in dilutions lower than this one may run into the effect of demonstrating a toxic effect which will be discussed in the paragraphs below

A fourth difference involves the difference in the yield of infectious virus in different HeLa cell systems with different adenovirus types It is demonstrated here with great ease that the sensitivity of the HeLa cell to the influence of the adenovirus is determined by the composition (80, 84) of the culture media Because cultures containing tryptophosphate broth usually show a steeper slope to the regression line than cultures without the broth It has been demonstrated (85) fairly effectively that there are inhibitors against adenoviruses in ox, in horse and in calf serum and these sera are frequently used in tissue culture media

A large number of workers have studied adenovirus infections of HeLa cells particularly the biochemical and morphological changes which may be induced in the cells It is of interest that viruses absorb onto HeLa cells (79, 82, 87) in the order of 4 to 6 hours which is relatively slow and that only a small proportion of the cells in a HeLa cell culture may be infected However, recently with a single stage increase in multiplication, evidence may be accumulating (86) that would indicate that a larger number of HeLa cells were actually infected than previously thought The latent period before increase in the titer in the intracellular virus may be anywhere from 14 to 21 hours (67, 88, 89) and varys with the different types of HeLa cells and with the virus type involved Several workers (82) have reported that the virus is higher intracellular than it is extracellular The

centrifuging technics have shown that most of the infectious virus is in the cytoplasm of the cell whereas the cytopathogenic and staining evidence would indicate that most of the viral replication takes place in the nucleus

Within 24 hours after inoculation there is seen in the nucleus of the HeLa cell a small osmiophilic body (82) which is usually surrounded by a clear zone and these are ordinarily Feulgen negative However as time passes these inclusions become basophilic and Feulgen positive (90, 91) and the nucleus is ultimately destroyed after it becomes enlarged and distorted The inclusions increase in size, become quite densely basophilic, and are a strongly Feulgen positive mass whereas the remainder of the nucleus is vaculoated to a large degree Sometimes the changes may be different with different types of adenovirus infection, for instance, types 3 and 4 produce inclusion bodies which appear crystal like particularly in the clear zone of the nucleus and there may with these 2 types be reticular masses which are sometimes partly Feulgen positive, and then become basophilic and produce a large Feulgen positive inclusion body The different kinds of changes by types 1, 2, 5 and 6 are seen to be essentially identical but those with 3, 4 and 7 are different from the first 4 viruses noted

There is a stimulation in the uptake of P 32 (92) and glycine by HeLa cells infected with adenovirus 2 within 2 hours after inoculation and in addition there is an increase in the production of lactic acid within 8 hours Other workers (93) have demonstrated that the increase in acid production was also accompanied by an increase in the utilization of oxygen along with an accumulation of alpha keto glutaric, pyruvic and acetic acids There are several fashions in which these authors try to explain this increase but they state that it may not be due altogether to viral synthesis but actually may be a type of non specific response which is seen in cellular injury

If one studies the infected HeLa cell with the electron microscope (71, 72, 73, 94, 95, 96), he is likely to see a characteristic type of change in the nucleus of the cell Shortly after infection there appears in the nucleus osmiophilic granules which are noted in amorphus type areas and the nucleus begins to undergo definitive changes which are capable of being measured Shortly after the formation of these particles and materials, there is a crystalline array of the virus particles formed in the nucleus and these seem to arrange themselves in a lattice like formation By ingenious use of sections from the same cells, workers have indicated that the initial amorphus material is

Feulgen negative whereas the crystals arraying themselves in lattice-like formation are the Feulgen positive inclusions.

Another factor which may be involved in the titration problem of adenoviruses in HeLa cells has to do with the formation of a cell detaching factor (106) which is formed by adenoviruses growing in tissue cultures. This cell detachment factor (CDF) is characterized by its ability to bring about the detachment of HeLa and RKB cells from the surfaces of the glass within 4 to 6 hours after inoculation. A method for the testing of the cell detachment material has recently been reported. The authors state that the CDF is smaller and is somewhat more resistant to the effect of heat and ultraviolet than is the infectious virus particle itself, further the material is trypsin sensitive and is nondialyzable. It is of interest that the antibodies which were capable of inhibiting the cell detachment could not be correlated in a quantitative fashion with either the homologous neutralizing antibody nor with the complement fixing group antibody shared by most of the adenoviruses. Also, the inhibition of the cell detachment was not observed unless these other antibodies were present in the serum.

In connection with the subgrouping of 7a, a recent report indicates that the minor antigen differences between the 7 and 7a viruses is so slight there is little need to suggest that the subgroup 7a should be employed, at least at this time.

Oncolysis with Adenoviruses

In the early stage of the development of the information with respect to this group of diseases, it became fairly obvious that since the agents were quite destructive to the different kinds of cells growing in tissue culture it might be of considerable interest to test the oncolytic effect by inoculating adenoviruses into human tumors both in patients and in laboratory animals. One report (97) indicates that in 26 of 40 cervical carcinomas inoculated directly or by intra arterial injection there was a local neucrosis. It seemed that this local necrotic response was more frequent if there was a homotypic antibody in the preinoculation serum. There is another report (98) by other workers to the effect that no oncolysis was noted with type 4 adenovirus given to 14 patients with incurable malignant tumors.

Adenoviruses inoculated in HeLa cell tumors, which were grown in the peritoneal cavity of rats which had received X ray and cortisone,

produced no oncolysis (99) and in general only a very small amount of virus was recovered There appears to be an inhibition of tumor growth with type 3 virus when it is mixed with the tumor suspension prior to inoculation in H Ep 3 tumor (100) as reported by other investigators

It is obvious that oncolysis has only begun to be studied with respect to adenoviruses and it may be that a specific virus may need to be ascertained for each specific patient in order to study this effect with greater accuracy

Epidemiology of Adenovirus Diseases

There have been several reviews (5, 48, 101, 102) of the epidemiology of these diseases and this report will not discuss this phase in considerable detail

It would appear that sex (34) does not have any influence of the childhood infections, however, it seems that type 4 infections are more prevalent in adult males (51) than in adult females on serologic evidence The age and geographic factors are somewhat important because there is a high prevalence of types 1, 2, 3 and 5 in infants and children whereas types 4, 7 and 14 are seen in military recruits in the United States and in Holland It would appear in some of the eastern countries, particularly in Japan, Saudi Arabia and Egypt that type 8 is quite prevalent in children Further studies along these lines need to be done It would appear that adenoviruses do not spread rapidly in schools as similar to the fashion in which Coxsackie, ECHO and polio viruses tend to spread, but the adenoviruses do spread readily in the home and they spread quite distinctively in orphanages, in hospitals and in summer camps It seems that the intimate type of exposure necessary for easier dissemination of some of the viruses is quite prevalent in military recruits centers

One of the epidemiologic features which should be emphasized has to do with the irritation of the mucous membranes, either those lining the nose or conjunctival sacs For instance, in the studies in volunteers (6) it was demonstrated that follicular conjunctivitis and pharyngoconjunctival fever could be produced definitively in adult susceptible individuals if one swabbed the virus onto the conjunctiva as noted above However, if one gave the virus by dropping it in the nose or spraying it, the disease was nothing like as definitive and

could not really be shown to be present in the volunteer On this same line swimming produces adenovirus conjunctivitis (28, 30, 31, 37, 38) and there may be a real role in corneal injury (103) in the etiology of EKC particularly with optomologic instrumentations (104 105) and with foreign bodies in industry and with the arc welding lamp exposure (103) All of these suggest that local irritation of the mucous membrane actually provides a means of the adenovirus to gain entry The incubation period for pharyngoconjunctival fever appears to be anywhere from 5 to 7 days with a range from 2 to 10 Infection of the volunteers (6) inoculated with various types of adenoviruses indicate that the incubation period is also in the order of 4 to 6 or possibly as long as 10 days

One of the interesting aspects of adenovirus infections has to do with the persistency of the virus In tissues of individuals infected however, it is of importance to note that in order to demonstrate these agents one must grow them *in vitro* (15 65) and that it is essentially impossible to implicate that the viruses may be present in the adenoid of the child as late as 8 to 10 or 12 years after the initial infection The duration of persistance is actually not known (61, 65) but one can judge that it is fairly high because of the extraordinarily high frequency of the same virus type from children and from adults It would appear that these persistent infections seen not only in children but in adults as well may provide the locus for the virus to reach epidemic proportions from this seeding which is constant throughout the population

The recently isolated myxovirus group has been defined and the members are stated (107) to have the following properties 1 hemag glutination of the red blood cells of fowls 2 associated with the virus is a receptor destroying enzyme (RDE) 3 the receptors are destroyed by RDE namely *Vibrio cholera* recepting destroying enzyme 4 the normal inhibiting serum may be removed by RDE or periodate 5 growth in the embryonic cavity of the chicken egg 6 a size of 80 to 150 millimicrons 7 ether sensitivity and 8 stability at —70° C Until a couple of years ago influenza A B and C viruses were the only members of this group of agents which were known to cause respiratory disease in humans However in the past 24 months, 4 new members have been demonstrated in persons with mild respir atory disease One of these namely Sendai virus is able to be isolated primarily by embryonic inoculation (108, 109) and propagates in eggs On the other hand the other 3 viruses namely CA and the 2 types

of HA virus (hemadsorption) are able to grow rather poorly in eggs (110, 111, 112) and tissue culture isolation is the method of choice

While the antigenic composition of the Sendai, CA and 2 types of HA viruses have not been completely characterized, it is apparent that they share a subgroup antigenic relationship (110, 113–116) with Newcastle disease virus and also mumps virus It is obvious that mumps virus has a common antigen or several antigens with Sendai virus and also with Newcastle virus

Recently it has been suggested (117) that these agents be reclassified as the parainfluenza group of myxoviruses Parainfluenza type 1 prototype becomes sendai and hemadsorption virus type 2, parainfluenza type 2 becomes CA virus and parainfluenza type 3 is HA, type 1 virus

Sendai (Parainfluenza 1)

The Sendai virus was recovered by Japanese workers (118) by the inoculation of mice intranasally who used autopsy material from cases of pneumonitis in infants There was some question as to whether these isolations were valid because identical agents (119–121) have been recovered recently from normal mice It seems however, that sufficient information is now at hand to support the contention of the original authors In the first place, the evidence is fairly conclusive that if this material is fed to a volunteer (118) there is a pneumonitis produced and further in another volunteer (122) a specific antibody level was produced to the agent in low titer The demonstration of the 5 criteria mentioned early in this review, have not been completed however

There was a fairly extensive outbreak reported (123) in Vladivostok in Russia which indicated that this agent was associated with influenza A One of the difficulties with this report however, has to do with the fact that the epidemic was not sufficiently studied from the laboratory point of view since only 5 virus strains were isolated and a total of 11 sets of sera were examined One rather interesting correlary to this epidemic was established (124) a year later by the examination of sera from individuals in different portions of Moscow and, also, from sera of individuals who had been ill in Vladivostok and it was demonstrated that the antibody to the Sendai virus was present in those individuals residing in Vladivostok whereas those in Moscow

contained no such antibodies This would suggest that the Sendai virus did not spread over any of the other Socialist republics in 1956 The number of isolations of Sendai virus leaves one with the serious question whether this agent is related to human disease However, an influenza like illness does seem to be produced in adults, and in young infants and in children the disease is characterized most frequently by a type of pneumonitis Sometimes there is an aseptic meningitis syndrome

In the laboratory the virus has been characterized as multiplying in the embryonic and in the allantoic cavities of the embryonated chicken egg (118) that it is capable of agglutinating guinea pig, human, type O, sheep, cow and chicken erythrocytes (125) and that it is approximately 150 millimicrons in size The receptors in the various cells have been shown to be sensitive to RDE, and the Sendai virus group has been shown to be antigenically related to mumps virus (116, 126) and also to the HA group Some authors (127, 128) have tended to report epidemics of respiratory diseases caused by the Sendai viruses on the basis of serologic evidence alone This is not acceptable evidence at the moment since primary isolation from the ill individuals would also be required in order to be certain that one was not dealing with an agent related antigenically to the Sendai viruses

Croup Associated Virus (CA, Parainfluenza Type 2)

The first report (110) of isolation of this agent was made in 1954 from infants with croup in Ohio using monkey kidney tissue culture This report was shortly followed by another from Canada (129) wherein the virus was isolated in HeLa cells and in human embryonic tissue cultures Approximately 50% of the infants studied in Cincinnati developed antibodies for this agent and two-thirds of the infants in Toronto yielded the virus by isolation technics The strains of virus in Toronto and Cincinnati and the strains of virus isolated in Canada and in Ohio have been shown to be identical A further isolation was recently demonstrated in the state of Iowa from a single infant Also children with a mild respiratory disease characterized by fever and aching yielded 5 viruses in the Washington, D C area Interestingly enough, the isolation in Ohio in Canada and in Washington, D C were made essentially at the same times indicating that the virus was disseminated over a wide area in the fall of 1955

One of the interesting properties of this agent in addition to its ability to hemagglutinate red cells and to grow rather poorly in the embryonic sac of the chicken embryo is a type of syncytial cyto pathogenic (110) effect which this agent is capable of producing in human amnion cultures and in monkey kidney It appears to be quite distinctive and oft times is similar to that which one sees with mumps virus, with which CA shares a common antigen Further study needs to be done with this group of agents and more isolates with more serum antibody studies should be carried out It would be of interest if the criteria mentioned in the early part of this review could be completed with respect to the CA group of agents

Hemadsorption Viruses (HA)

Two types of this group of agents have been recovered (111) during the recent years Type 1 was recovered from 55 children, 8 of these children were suffering from either pharyngitis or broncheol itis or pneumonia and were all hospitilized Twenty seven of the individuals from whom type 1 virus was recovered were involved in an outbreak of respiratory disease in a nursery The group of agents get their name from the fact that when guinea pig cells are added to the monkey kidney tissue culture the guinea pig red blood cells are adsorbed to the monkey kidney cells giving a typical hemadsorption

tion technic, many viruses might have been missed However, one should note that the hemadsorption phenomena (130) is characteristic not only of these agents but is also characteristic of other members of the myxovirus group as well Also, one need not use only guinea pig cells but the red blood cells from a variety of other species (132) may also be employed

Volunteer studies (131) with HA type 2 yielded interesting results in that an incubation period longer than the usual one in the experience of the authors led to the release of the volunteers at the sixth day after inoculation and within 24 hours, 8 of the individuals inoculated with HA type 2 had become ill Reisolation of the volunteers followed this episode and characteristic disease with isolation of the virus and an increase in serologic titer resulted Interestingly enough, approximately 6 days after the men in the study became ill, 8 other

individuals in the institution not in the study were ill with hemadsorption type viruses as shown by isolation and serum antibody rises in these individuals.

Type 1 and type 2 viruses are distinct from one another and neutralizing tests indicate that they are not particularly related except that they share a complement fixing antigen. The HA agents are related to Sendai viruses but they are antigenically distinct from influenza A, B and C, from CA, Newcastle viruses and from mumps.

Other New Respiratory Viruses: Respiratory Syncytial Virus (CCA)

This agent was first isolated from chimpanzees (133) and the initials CCA stand for "Chimpanzee Coryza Agent." However, the name respiratory syncytial was suggested recently since CCA would appear to be too restrictive as these agents are actually involved in human infection. The term respiratory syncytial indicates the fact that the agent is associated with respiratory tract disease and that the syncytial type of cytopathogenic effect is its predominant effect produced in tissue culture.

One animal of 14 chimpanzees yielded this agent in the initial report concerned with the announcement of the discovery of this new virus. The 13 remaining chimpanzees had an antibody to the virus in their bloodstream after they became well. If one inoculates chimpanzees intranasally with tissue culture material, there are coryza, rhinitis and other common cold type signs observed after approximately 5 days incubation period. The first report wherein these agents were concerned with human disease came with the isolation of the material from infants with croup and pneumonia.

The virus is widespread in that if one tests for the neutralizing antibody (134) one finds that half of the children under 2 years of age possess this antibody and approximately three fourths of the children at the age of 5 years have been exposed to this antigen. Further, it was demonstrated with serologic methods that the respiratory syncytial agent was causing infections in infants and small children in Maryland (134) in the winter of 1956 and 1957. Antibody specific for this group developed in 7% of individuals attending an outpatient dispensary when tested by paired serum technics, the same study stated that 45% of the children who were hospitalized for noninfectious diseases yielded a rise in neutralizing antibody. In the far East recently, evidence has been obtained (135) which indicates that

13% of a respiratory disease which is not influenza like and was not caused by adenoviruses occurred among the United Nations troops and the etiologic agent was thought to be related to CCA

Further study needs to be done with the respiratory syncytial group of agents and it would be helpful if volunteer studies could be done to assist in the clinical characterization of the disease

*JH and 2060 Viruses**

Recently newly recognized viruses have been recovered from persons with quite mild respiratory disease and sufficient data seems to be collected to warrant their inclusion in this review

The first viruses were isolated (136) from approximately 20 individuals of all ages and in all seasons of the year and these illnesses were characterized by malaise, by coryza, a mild sore throat and a very low grade fever These individuals were diagnosed as ordinary common colds and the authors state in their first report that approximately 20% of all individuals over the age of 8 years have neutralizing antibodies in their serum One of the difficulties with this agent was the fact that as it was isolated in monkey kidney tissues it required several passages before typical cytopathogenic effects were demonstrable in most laboratories In the early report, sufficient evidence was not forthcoming of an epidemiologic nature to accept the JH virus as the cause of respiratory illness

In a subsequent paper (137) a vaccine prepared in monkey kidney cultures was reported and it was stated that neutralizing antibodies were produced by this vaccine and further that the vaccine prevented illness due to this virus There were 50 vaccinated and 50 unvaccinated control children and 26 common cold type illnesses were seen in the study group It is of extraordinary interest that 23 of the 26 individuals occurred in the controls and there were only 3 were in the vaccinated group The neutralizing antibody rise was of a fourfold greater nature in 20 of the 26 individuals

Further work in other laboratories on this agent have indicated that by repeated passage in monkey kidney, one may be able to produce a type of disease in volunteers, in some laboratories (138), but in other laboratories (139) the results leave a good deal to be

* Editors' note The "2060" virus has been found to possess the properties of an enterovirus, and has been classified as the prototype strain of *ECHO virus*, type 28

desired In the next few years, the data to support or disprove whether JH is related to mild respiratory diseases will be forthcoming

The 2060 agent was isolated (140) at the Naval Medical Research Unit No 4 in Great Lakes, Illinois from Naval recruits in 1954 This agent has been shown to be related by human volunteer studies (138) to the JH virus, it grows slowly in monkey kidney cultures and the monkey kidneys often must be prepared in a special fashion in order to secure the passage of the agent One of the problems with this agent has been that it too fails to produce disease in volunteers in one laboratory (141) and does produce disease in the hands of other workers (139) The serologic surveys conducted by the original authors indicate an increase in neutralizing antibody with increasing age of children

The original authors conclude that the evidence supporting an etiologic relation to respiratory disease is circumstantial and they did not rule out the possibility of a coincidental infection

One of the primary problems with studying the 2060 and the JH agent has to do with production of a suitable antigen for carrying out serologic procedures in the study of these diseases in humans Also, a reference serum of good potency would be helpful.

Coe Virus

This agent was isolated in California (142) in recent years and has been demonstrated by serologic and by isolation technics to be related to mild respiratory disease Sufficient data on this virus has not been carried out however to justify its acceptance as yet as a new member of the respiratory virus group

Summary

With the advent of new technics, of tissue culture, with the use of hemadsorption procedures, the development of the complement fixation and neutralizing antibody technics, the respiratory virus field in the past 4 years has begun to be clarified more than any time in the history of medical science The criteria mentioned in the early part of this review, if carried out, are definitive for the establishment of new agents of disease and would, if utilized, lead to somewhat less confusion than is currently available with respect to some of the newer agents

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The technics for diagnosing these diseases are fairly simple and there is no reason why they should not be employed in the routine hospital diagnostic laboratory to assist the clinician in determining the role of the new agents in reproduction of specific diseases

One aspect of this group of diseases which has not been emphasized in this review is the clinical nature of the illnesses produced It cannot be emphasized sufficiently however, that the illnesses should be defined very carefully by good clinical studies because only in this fashion can one hope to secure adequate information to define the illness problems in the field of mild respiratory disease

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